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(54) Title: EXPRESSION SYSTEMS FOR PREPARATION OF POLYPEPTIDES IN PROKARYOTIC CELLS (57) Abstract Expression cassettes for enhanced expression and production of a polypeptide of interest in prokaryotic cells are provided. The expression cassettes provide for production of the polypeptide of interest so that such polypeptide can either be secreted from the host cell in an active conformation or conveniently processed and renatured to a functional state. Preferably, the polypeptide of interest is expressed as a fusion protein, particularly fused to a leader sequence from a highly expressed bacterial or bacteriophage-gene. The polypeptide of interest may subsequently be cleaved from the leader sequence and refolded, or used as a fusion protein.		

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5 **EXPRESSION SYSTEMS FOR PREPARATION
OF POLYPEPTIDES IN PROKARYOTIC CELLS**

CROSS-REFERENCE TO RELATED APPLICATION

10 This application is a continuation-in-part of
U.S. Application Serial No. 240,768, filed September 2,
1988 which is a continuation-in-part of U.S. Appli-
cation Serial No. 115,139, filed October 30, 1987,
which disclosures are hereby incorporated herein by
reference.

15

INTRODUCTION

Technical Field

20 This invention relates to compositions and
methods for preparation of novel polypeptides, in
particular fusion polypeptides, using recombinant DNA
techniques.

Background

25 The advent of genetic engineering brought with
it the promise of easy production of large quantities
of a variety of peptides. However, this promise has
not been fully realized for a number of reasons. For
example, in many instances where the peptide has been
30 produced and retained in the cytoplasm of the host
organism, inclusion bodies have resulted requiring
denaturation and renaturation of the protein, frequent-
ly with only partial or little success. In other
instances, the peptide has been substantially degraded
35 so that not only are yields low, but also complicated
mixtures are obtained, which are difficult to
separate.

As a potential solution to these difficulties, the possibility of obtaining secretion of a desired peptide into the nutrient medium has been investigated. Obtaining secretion of the desired protein has met with limited success in the past, since not all proteins are capable of being secreted by the host cells which have been employed. Moreover, even when secreted, the processing of the peptide by the host cell may result in a product which differs from the composition and/or conformation of the desired polypeptide and the yields of protein have been less than expected. There is, therefore, a substantial interest in developing systems for the efficient and economic production of active peptides where the desired polypeptide can accumulate in the host cell without degradation and can either be secreted in an active conformation or conveniently processed and renatured to a functional state.

20

SUMMARY OF THE INVENTION

Expression cassettes, and methods for their preparation and use are described, which provide for enhanced expression and production of an active gene product. The expression cassettes include efficient transcriptional and translational initiation and termination regulatory regions appropriate for the host cell to provide for expression of a desired polypeptide. The expression cassette preferably further includes, as appropriate for the host cell, a leader sequence for expression under the transcriptional and translational regulation of the regulatory region, sequences providing for enzymatic or chemical cleavage sites for cleavage of the leader peptide from mature polypeptide, and regulatory sequences which allow the time of expression of the gene of interest to be modulated. The expression cassettes are introduced into a host cell under conditions whereby the resulting

transformants stably maintain the expression cassette. Naturally occurring DNA and synthetic genes may be employed for the production of a polypeptide of interest.

5

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, expression cassettes are provided which, when inserted into a host cell, allow for the preparation of a polypeptide of interest which has enhanced stability and is either secreted in an active conformation or may be conveniently processed and renatured to an active state.

To obtain increased expression of a polypeptide of interest in a host cell, the nucleotides encoding the N-terminal amino acids of the polypeptide of interest are modified within the constraints of codon degeneracy to mimic those of the natural gene sequence found with the Shine-Dalgarno sequence used in the expression cassette. The expression cassette thus will have the following general structure:

P--S.D.--met--G₁

wherein

P comprises a promoter sequence including the regulatory regions occurring at about -35 and -10 nucleotides upstream from the start of the RNA chain and may also include regulatory sequences allowing for the induction of regulation;

S.D. comprises a Shine-Dalgarno sequence; met comprises a codon for the initiating methionine of the polypeptide of interest; and

G₁ comprises the gene for the polypeptide of interest wherein the first 7 to 30 codons of the gene have been modified wherever possible, using codon degeneracy to approximate the nucleotide sequence of the natural gene which would follow the Shine-Dalgarno sequence used in the expression cassette.

As an alternate means of obtaining increased expression of the polypeptide of interest from the host cell, the polypeptide can be expressed as a fusion protein by including in the expression cassette a DNA sequence encoding a leader sequence peptide joined in reading frame upstream from the gene of interest. Expressing the polypeptide of interest as a fusion protein can result in up to 30% or more of the protein produced by the host cell being the polypeptide of interest. The expression cassette for expressing a fusion protein will thus have the following basic structure:

P--S.D.--met--L--G

wherein:

P, S.D. and met have the meaning described above;

G comprises a gene for the polypeptide of interest; and

L comprises a DNA sequence encoding a leader peptide which may be an N-terminal sequence from any bacterial or bacteriophage gene, but generally is from a highly expressed gene; an amino acid sequence containing large numbers of hydrophobic amino acid residues; or an amino acid sequence containing large numbers of hydrophilic amino acid residues. When L comprises a hydrophobic amino acid sequence, this sequence will preferably also function as a signal sequence, allowing secretion of the polypeptide of interest from the host cell and cleavage of the signal sequence from the polypeptide. The DNA sequence coding for L may also be modified wherever possible, using codon degeneracy to approximate the nucleotide sequence of the natural gene which would follow the Shine-Dalgarno sequence used in the expression cassette.

The expression cassette described above provides for a fused expression product comprising the leader peptide and the polypeptide of interest. If it

is desired to obtain the polypeptide of interest alone and there is no convenient cleavage site, e.g. as provided by a natural signal sequence, a cleavage site may be provided for by joining at least one codon encoding a cleavage site in reading frame upstream from the gene of interest. The cassette will thus have the following structure:

P--S.D.--met--L--C--G

wherein P, S.D., met, L and G have the meaning described above and

C comprises at least one codon providing for a chemical or enzymatic cleavage site.

To stabilize the mRNA and to provide for higher levels of expression of a desired polypeptide, a transcriptional termination region (T) can be included in the expression cassette downstream from the gene of interest. An example of an expression cassette comprising T is as follows:

P--S.D.--met--G--T

although T may be included in any of the expression cassettes as described above.

Construction of Expression Cassettes

Design of an expression system to yield high levels of gene product must take into consideration not only the particular regions of a gene which have been determined to influence expression but also how these regions (and thus their sequences) influence each other. Where possible, choice of appropriate regulatory sequences will take into account the various factors which affect expression. Different genes have evolved a combination of all of these factors to yield a particular rate of expression; thus highly expressed genes can be considered useful models.

In terms of transcriptional regulation, the amount and stability of messenger RNA are important factors which influence the expression of gene

products. The amount of mRNA is determined by the copy number of a particular gene, the relative efficiency of its promoter and the factors which regulate the promoter, such as enhancers or repressors. Initiation
5 is believed to occur in the region just upstream of the beginning of the coding sequence.

The promoter in prokaryotic cells comprises nucleotide sequences which can affect the efficiency of transcription. These sequences include the regulatory
10 regions at about -35 and -10 nucleotides from the start of the RNA chain. Efficient promoters include those in which the nucleotide sequence of the -35 and -10 regulatory regions is substantially the same as consensus sequences for these regions in bacterial
15 promoters from highly efficient genes. Generally these regions are about 5 nucleotides and 6 nucleotides, respectively, in length, and each sequence may vary by about 1 nucleotide in length and/or in sequence. A preferred sequence for the -35 consensus regulatory
20 sequence is from the trp promoter, namely TGACA, and for the -10 consensus regulatory sequence is from the lac promoter, namely TATAAT.

Not only the nucleotide sequences but also the spacing of the consensus sequences of the -35 and -10
25 regulatory regions, with respect to each other, is important for obtaining optimum transcription of the gene of interest. Generally, the consensus sequences of the -35 and -10 regulatory regions are separated by about 16 to 18 nucleotides, preferably by about 17
30 nucleotides.

Illustrative transcriptional regulatory regions or promoters which provide for efficient transcription include the β -gal promoter, lambda left and right promoters, the trp and lac promoters and trp-lac (tac)
35 fusion promoters, and the like. Synthetic promoters having sequences substantially similar to these sequences may also find use. A preferred promoter is a

fusion promoter comprising the -35 regulatory region from the trp promoter and the -10 regulatory region from the lac promoter. Most preferably, the promoter is one in which the -35 trp consensus sequence is located about 17 nucleotides upstream from the -10 lac consensus sequence.

The transcriptional regulatory region may additionally include regulatory sequences which allow the time of expression of the gene of interest to be modulated, for example by presence or absence of nutrients or expression products in the growth medium, temperature, and the like. For example, expression of the gene of interest may be regulated by temperature of the host cell growth medium by including a regulatory sequence comprising the bacteriophage lambda P_L promoter, the bacteriophage O_L operator and the gene CI857 which codes for the temperature-sensitive C_I repressor in the expression vector. This would allow regulation of the promoter by interaction between the repressor and the operator at low temperatures, for example about 30°C. Increasing the temperature to about 42°C would inactivate the repressor and allow expression of the gene of interest.

As an example of modulation using growth-medium nutrients, regulation of the lac or the trp-lac hybrid promoter can be accomplished by use of the gene for the LacI repressor, which binds in the lac promoter region downstream from the -10 regulatory region. The LacI repressor gene may be present on an episome, preferably the lacIq enhanced mutant, or can be included in the expression cassette itself. Presence of a significant concentration of the repressor in the growth medium inhibits the promoter function in the absence of inducers. Thus addition of IPTG or lactose to the host cell growth medium enhances promoter function. When the bacterial strain is Lac⁺, lactose may be used as an inducer instead of IPTG.

The transcriptional regulatory region may additionally include regulatory sequences which terminate transcription and which provide sequences or structures which inhibit degradation of the mRNA and thus increase the stability of the mRNA species and allow for higher expression. Several examples of prokaryotic sequences are known -- the trp terminator, the gene 32 (T4) terminator, or synthetic terminators which are similar in sequence to gene 32.

10 In terms of translational regulation, given the presence of mRNA, expression can be regulated by influencing the rate of initiation (ribosome binding to the mRNA), the rate of elongation (translocation of the ribosome across the mRNA), the rate of post-
15 translational modifications and the stability of the gene product. The rate of elongation is probably affected by codon usage; the use of codons for rare tRNA's may reduce the translation rate. It is therefore preferable to use codons which frequently
20 appear in genes normally expressed by the host cell to increase the translation rate.

Downstream from the -35 and -10 regulatory regions is a consensus nucleotide sequence, generally AGGA, termed the Shine-Dalgarno sequence, which is
25 believed to be involved in ribosomal binding. Optimum ribosomal binding and initiation of translation can be achieved by using a ribosomal binding site functional in the host cell from a highly expressed gene. *
Evidence also points to the presence of nucleotide
30 sequences surrounding the Shine-Dalgarno sequence and sequences within the coding region which can affect ribosome binding, possibly by the formation of structural motifs through which the ribosome recognizes the initiation site, thus altering nucleotide sequences
35 of the coding region can be used to achieve optimum ribosomal binding and initiation of translation. The sequence of the first about 7 to 30 codons after the

initiating codon ATG can also affect binding and expression. Preferably the leader sequence and the Shine-Dalgarno sequence are obtained from the same gene, or where they are obtained from different genes, the codons of the leader sequence can be modified using codon degeneracy to approximate the nucleotide sequence of the natural gene that follows the leader sequence.

The position of the AGGA sequence with respect to the initiating ATG codon can influence expression. Generally the Shine-Dalgarno sequence is located from about 5 to 9 nucleotides from the initiating codon, although, unexpectedly, high levels of expression can be achieved using expression cassettes wherein the Shine-Dalgarno sequence is located from about 10 to 13 nucleotides, preferably 11 to 12 nucleotides from the initiating codon.

Stability of the mRNA is governed by the susceptibility of the mRNA to ribonuclease enzymes. In general, exonuclease digestion is inhibited by the presence of structural motifs at the ends of the mRNA; palindromic structures, altered nucleotides or specific nucleotide sequences. Endonuclease digestion is believed to occur at specific recognition sites within the mRNA and stable mRNA would lack these sites. There is also some evidence that mRNAs undergoing high levels of translation are also protected from degradation by the presence of ribosomes on the mRNA.

Stability of the expression product is aided by expression of the desired gene product as a fused polypeptide in which the desired polypeptide is expressed in conjunction with a second polypeptide or fragment thereof, especially a bacterial polypeptide. Preferably, stability of the expression product is achieved by providing for synthesis of a fusion protein in which the polypeptide of interest is expressed, joined to a leader sequence. A DNA sequence encoding an N-terminal amino acid sequence from, for example, a

highly expressed bacterial or bacteriophage gene such as the bacteriophage lambda N protein gene or cro gene or the β -galactosidase gene is joined upstream from and in reading frame with the gene of interest. The leader
5 sequence usually includes from about 8 to about 35, preferably from about 15 to about 25, N-terminal amino acids.

Expression of the polypeptide of interest as a fused protein with a leader sequence from another gene
10 has several advantages in addition to providing for stability. For example, the presence of the N-terminal amino acids provides a means for using general purification techniques for purification of any of a variety of polypeptides. For example, the N-terminal amino
15 acids of the N-protein are predictably antigenic, and thus specific antibodies raised against the N-terminal amino acids of the N-protein may be used for the amino purification of the fusion proteins containing the N-terminus of the N-protein. Furthermore, the N-terminus
20 of the N-protein has a high positive charge, which facilitates purification of the desired protein by ion-exchange chromatography, and the like.

The leader sequence can also be a hydrophobic amino acid sequence, which may additionally function as
25 a signal sequence for secretion. A DNA sequence encoding the signal sequence is joined upstream from and in reading frame with the gene of interest. Typically, the signal sequence includes a cleavage site which is recognized by a signal sequence peptidase.
30 Thus, positioning the polypeptide of interest directly after the signal sequence cleavage site will allow it to be specifically cleaved from the signal sequence and secreted as a mature polypeptide. Examples of hydrophobic amino acid sequences include the bacterial
35 alkaline phosphatase signal sequence; the OMP-A,B,C,D,E or F signal sequences; the LPP signal sequence, β -lactamase signal sequence; and toxin signal sequences.

Other leader sequences which can be used include hydrophilic sequences, for example the N-terminal 41 amino acid residues from amphiregulin which may provide for modification of the function of the polypeptide of interest. In addition, a cytotoxic agent such as a toxin A-chain fragment, ricin A-chain, snake venom growth arresting peptide, or a targeting molecule such as a hormone or antibody can be coupled covalently with the leader sequence with in most cases minimal effect on the biological activity of the gene product of interest. As with the other leader sequences, a DNA sequence encoding the leader sequence is joined upstream from and in reading frame with the gene of interest.

Where the leader sequence is not a signal sequence or does not contain a convenient natural cleavage site, additional amino acids may be inserted between the gene of interest and the leader sequence to provide an enzymatic or chemical cleavage site for cleavage of the leader peptide, following purification of the fusion protein, to allow for subsequent purification of the mature polypeptide. For example, introduction of acid-labile aspartyl-proline linkages between the two segments of the fusion protein facilitates their separation at low pH. This method is not suitable if the desired polypeptide is acid-labile. The fusion protein may be cleaved with, for example, cyanogen bromide, which is specific for the carboxy side of methionine residues. Positioning a methionine between the leader sequence and the desired polypeptide would allow for release of the desired polypeptide. This method is not suitable when the desired polypeptide contains methionine residues.

Where the leader sequence comprises a signal sequence, genes of interest with secretory leader sequences can be expressed with or without the leader sequence under conditions where the sequence may be

retained or cleaved. In addition, to obtain a high proportion of the desired polypeptide as a mature, cleaved and refolded peptide secreted into the medium, it is preferable to use a promoter, such as the tac promoter, which can operate at a lower temperature, for example about 30°C. Unexpectedly, higher levels of secretion can be obtained at the lower temperatures. Extremely high expression levels can prevent full translational modifications of the protein to occur, resulting in aggregation and accumulation of uncleaved precursor (i.e., structural protein and secretory leader). Similarly, growth at elevated temperatures, for example 42°C, also tends to result in aggregation and accumulation of uncleaved precursor.

The polypeptide of interest may be any polypeptide for which expression is desired and may be either homologous (derived from the host cell) or heterologous (derived from a foreign source or synthetic DNA sequence). The polypeptide may be derived from prokaryotic sources, or eukaryotic sources, which eukaryotic sources may include fungi, protists, vertebrates, invertebrates, and the like. The polypeptide of interest may include enzymes such as isopenicillin synthetase; mammalian peptides such as interleukins, cytokines, growth factors, e.g. epidermal growth factor, platelet-derived growth factor, oncostatin M, TGF- α and - β , viral growth factors, e.g. Vaccinia Virus, Shope's fibroma; snake venom growth-arresting peptide, brain-derivable peptides, immunoglobulins and fragments thereof, and the like.

Where the gene of interest is to be expressed in a host which recognizes the natural transcriptional and translational regulatory regions of the desired gene of interest, the entire gene with its natural 5' and 3'-regulatory regions may be introduced into an appropriate expression vector. However, where the gene is to be expressed in a host which recognizes the

natural transcriptional and translational regulatory regions less well, further manipulation may be required. The non-coding 5'-region upstream from the gene of interest may be removed by endonuclease restriction, Bal31 resection, or the like.

Alternatively, where a convenient restriction site is present near the 5'-terminus of the gene of interest, the gene of interest may be restricted and an adapter employed for linking the gene of interest to the promoter region, where the adaptor provides for the lost nucleotides of the gene of interest. A variety of 3'-transcriptional regulatory regions are known and may be inserted downstream from the stop codons.

The DNA sequences encoding the polypeptide of interest can be synthesized using conventional techniques giving overlapping single strands which may be ligated together to define the desired coding sequences. The termini can be designed to provide restriction sites or one or both termini may be blunt-ended for ligation to complementary ends of an expression vector. For expression of the sequence an initiating methionine is provided. Expression vectors are generally available and are amply described in the literature.

Instead of synthesizing the gene of interest, the gene may be isolated by various techniques. These include isolating mRNA from a host organism which codes for the polypeptide of interest, the mRNA reverse transcribed, the resulting single-stranded (ss) DNA used as a template to prepare double-stranded (ds) DNA and the ds DNA isolated. Another technique is to isolate a piece of the host cell genomic DNA, and using a probe, appropriately degenerate, comprising a region of the most conserved sequences in the gene encoding the polypeptide of interest, identify sequences encoding the polypeptide of interest in the host cell genome. The probe can be considerably shorter than the

entire sequence, but should be at least 10, preferably at least 14, more preferably at least 20 nucleotides in length. Longer oligonucleotides are also useful, up to the full length of the gene encoding the polypeptide of interest. Both DNA and RNA probes can be used.

In use, the probes are typically labeled in a detectable manner (for example with ^{32}P -labelled or biotinylated nucleotides) and are incubated with single-stranded DNA or RNA from the organism in which a gene is being sought. Hybridization is detected by means of the label after single-stranded and double-stranded (hybridized) DNA or DNA/RNA have been separated, typically using nitrocellulose paper. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art.

Although probes are normally used with a detectable label that allows for easy identification, unlabeled oligonucleotides are also useful, both as precursors of labeled probes and for use in methods that provide for direct detection of DNA or DNA/RNA. Accordingly, the term "oligonucleotide" refers to both labeled and unlabeled forms.

Once the desired DNA sequence has been obtained it may be manipulated in a variety of ways to provide for expression. For example, chimeric polypeptide sequences may be prepared by combining gene fragments of at least two polypeptides having sequences substantially similar to naturally occurring polypeptide chains. It is highly desirable that the three dimensional structure of the polypeptide be retained, particularly that portion of the structure which may be responsible for biological activity of the polypeptide of interest. Depending upon the source of the fragments and the length of the desired polypeptide, convenient restriction sites may be designed into the synthetic genes used to construct the

chimeric polypeptides. When possible the restriction site(s) leaves the amino acid sequence of the polypeptide unaltered. However, in some cases incorporation of the new restriction site(s) may yield an altered amino acid sequence without changing the activity of the protein.

During the construction of the expression cassette, various fragments of the DNA will usually be cloned in an appropriate cloning vector, which allows for amplification of the DNA, modification of the DNA or manipulation by joining or removing of sequences, linkers, or the like. Normally, the vectors will be capable of replication in at least a relatively high copy number in bacteria. A number of vectors are readily available for cloning in gram-negative bacteria, especially E. coli, including such vectors as pBR322, pACYC184, M13, Charon 4A and the like. The cloning vectors are characterized by having an efficient replication system functional in the host bacteria.

The cloning vector will have at least one unique restriction site, usually a plurality of unique restriction sites and may also include multiple restriction sites. In addition, the cloning vector will have one or more markers which provide for selection of transformants. The markers will normally provide resistance to cytotoxic agents such as antibiotics, heavy metals, toxins or the like, complementation of an auxotrophic host, or immunity to a phage. By appropriate restriction of the vector and the cassette, and, as appropriate, modification of the ends, by chewing back or filling in overhangs, to provide for blunt ends, by addition of linkers, by tailing, complementary ends can be provided for ligation and joining of the vector to the expression cassette or component thereof.

After each manipulation of the DNA in the development of the cassette, the plasmid will be cloned and isolated and, as required, the particular cassette component analyzed as to its sequence to ensure that the proper sequence has been obtained. Depending upon the nature of the manipulation, the desired sequence may be excised from the plasmid and introduced into a different vector or the plasmid may be restricted and the expression cassette component manipulated, as appropriate.

In some instances a shuttle vector will be employed where the vector is capable of replication in different hosts requiring different replication systems. This may or may not require additional markers which are functional in the two hosts. Where such markers are required, these can be included in the vector, where the plasmid containing the cassette, two replication systems and the marker(s) may be transferred from one host to another, as required. For selection, any useful marker may be used. Desirably, resistance to neomycin or tetracycline are of interest. However, although a marker for selection is highly desirable for convenience, other procedures for screening transformed cells have been described. See for example G. Reipin et al. Current Genetics (1982) 189-193. Transformed cells may also be screened by the specific products they make, for example, synthesis of the desired product may be determined by immunological or enzymatic methods.

The expression cassette may be included within a replication system for episomal maintenance in an appropriate cellular host or may be provided without a replication system, where it may become integrated into the host genome. The DNA may be introduced into the host in accordance with known techniques, such as transformation, using calcium phosphate-precipitated

DNA, transfection by contacting the cells with a virus, microinjection of the DNA into cells, and the like.

Once the gene of interest has been introduced into the appropriate host, the host may be grown to express the gene of interest. A variety of prokaryotic hosts may be employed. Host cells can include gram-negative organisms such as E. coli, e.g., JM109, JM101, and 107; HB101, DH1 or DH5. Particularly suitable are gram-positive organisms such as B. subtilis which have no periplasmic space and directly secrete polypeptides into the growth medium.

The host cell may be grown to high density in an appropriate nutrient medium. Where the promotor is inducible, permissive conditions will then be employed, for example, temperature change, exhaustion or excess of a metabolic product or nutrient, or the like. For example, where the regulatory sequence comprises the bacteriophage λP_L promoter, the bacteriophage O_L operator, and the CI857 temperature sensitive repressor, the host cells may be grown at the permissive temperature, generally about 30°C, at which temperature transcription from the P_L promoter is repressed and the host cells may grow unhindered by the demands of the synthesis of the foreign gene product, which additionally may be toxic to the host organism. When the host cells have reached an optimal density, the temperature may be increased to a non-permissive temperature, for example about 42°C, at which time the CI repressor is rendered inactive, permitting transcription from the P_L promoter.

Maximal secretion can be obtained by using the lac promoter or a trp-lac promoter and induction with a metabolic inducer such as lactose for a lac^+ host strain, and providing $lacI^Q$ on a vector. Examples of host cells which could be used with this system include DH1, DH5 or HB101.

Where the product is retained in the host cell, the cells are harvested, lysed and the product isolated and purified by extraction, precipitation, chromatography, electrophoresis, and the like. Where
5 the product is secreted into the periplasmic space, the cells are harvested and the product is liberated by destruction of the cell wall, e.g., by hypotonic shock and the like. Where the product is secreted into the medium, the nutrient medium may be collected and the
10 product isolated by conventional means, for example, affinity chromatography. To produce an active protein it may be necessary to allow the protein to refold. If the protein is expressed as a fusion protein with the leader sequence, the leader sequence may be removed by
15 treatment with for example formic acid or cyanogen bromide. The leader sequence preferably is removed after refolding of the protein.

The following examples are offered by way of
20 illustration and not by way of limitation.

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30

35

EXPERIMENTALTable of ContentsBiological Deposits

5

MethodsExample I.Activity Assays

10

- A. Mitogenic Assay
- B. Soft Agar Colony Growth Stimulation Assay
- C. EGF Receptor Binding Inhibition Assay
- D. Wound Healing

15

- 1. Mid-Dermal Thermal Injuries
- 2. Mid-Dermal Donor Graft Injuries
- E. Inhibition of DNA Synthesis
- F. Inhibition of Tumor Growth in Nude Mice

20

Example IIConstruction of Cloning and Expression Plasmids

A. Plasmid pBM11

25

- 1. Construction of pBM4
- 2. Construction of pBM8
- 3. Construction of pBM9
- 4. Construction of pBM10
- 5. Final Construction of pBM11

B. Construction of pBM11M4

30

- 1. Construction of pBM11/M
- 2. Construction of pBM11/M1
- 3. Construction of pBM11/M2
- 4. Construction of pBM11/M3
- 5. Final Construction of pBM11/M4

C. Construction of pBM11M5

35

- D. Construction of pBM11/C Series
- E. Construction of pBM11/NDP

- F. Construction of pBM16t
- G. Construction of pBM16/NDP
- H. Construction of pBM11/PAD (also called pBM11M3/PAD)
- 5 I. Construction of pBM14
 - 1. Construction of pBM12
 - 2. Construction of pBM13
 - 3. Final Construction of pBM14
- 10 J. Plasmid pLEBam
- K. Plasmid plac/cro- β gal
- L. Plasmid ptac/cro- β gal
- M. Plasmid TacPak
- 15 N. Plasmid pTCPt
- O. Plasmid pTNPt
 - 1. Preparation of pBM16t
 - 2. Preparation of the 2.8kb EcoRI-BamHI fragment of pBM16t/VGFa lacking the HindIII site
 - 20 3. Preparation of the 150bp BamHI-BsmI fragment of pBM11/PAK
 - 4. Preparation of oligonucleotides TacA+ and TacA-
 - 5. Ligation and Isolation of pTNPt

Example III.

25

Preparation of Genes of Interest.

- A. Synthetic Growth Factor Genes
 - 1. TGF Synthetic Oligonucleotides
 - 2. VGF Synthetic Oligonucleotides
 - 30 3. EGF Synthetic Oligonucleotides
 - 4. Assembly Growth Factor Genes
- B. Synthetic Platelet Factor 4 Gene
- C. DNA Cloning of Oncostatin M
 - 35 1. Preparation of cDNA Libraries
 - 2. Restriction Site Mapping
 - 3. DNA Sequence of Oncostatin M
 - 4. RNA Analysis

Example IV.Expression of the Polypeptide of Interest
as a Fusion Protein with the N-Protein

- 5 A. Modified Synthetic TGF
1. Preparation of pBM11/N/TGF
- B. Modified Synthetic TGF-VGF Hybrid
1. Preparation of pBM11/N/TTV
- 10 C. Synthetic Platelet Factor 4
1. Preparation of pBM11/N/PF4

Example V.

15 Expression of the Polypeptide of Interest
 as a Fusion Protein
 with the N-Protein and a Cleavage Site

- A. Modified Synthetic VGF
1. Preparation of pBM11/NDP/VGFA
- 20 2. Preparation of pBM11/NDP/VGFa
- B. Modified Synthetic TGF-VGF Hybrids
1. Preparation of pBM11/NDP/TTV
2. Preparation of pBM11/NDP/VTV
3. Preparation of pBM16/NDP/TVV
- 25 C. Synthetic EGF
1. Preparation of pBM11/NDP/EGF
- D. Synthetic Platelet Factor 4 (PF4)
1. Preparation of pBM11/NDP/PF4
- 30 E. Oncostatin M
1. Construction of pBM16/NDP/OncoM
2. Preparation of pBMX/OncoM

Example VI.

Expression of the Polypeptide of Interest
As a Fusion Protein
with the Modified Alkaline Phosphatase Signal Sequence

- 5 A. Preparation of pBM11/PAD/EGF
1. Preparation of 0.17kb EcoR I-BamHI fragment of EGF
 2. Preparation of 0.5kb Pvu I-Hind III fragment of pBM11/PAD
 - 10 3. Preparation of the 5.2kb Pvu I-BamHI fragment of pBM11/PAD
 4. Ligation and isolation of pBM11/PAD/EGF
- B. Preparation of pBM11/PAD/OncoM
1. Preparation of modified Oncostatin M gene fragment
 2. Preparation of pBM11M3/PAD fragments
 - 15 3. Ligation and isolation of pBM11/PAD/OncoM
- C. Preparation of pBM11/PAD/nVGf α
1. Preparation of 0.5kb HindIII-PvuI digested pBM11/PAD
 2. Preparation of the 5.2kb PvuI-BamHI pBM11 plasmid fragment
 - 20 3. Preparation of the 170bp NcoI(blunt)-BamHI synthetic VGf α gene
 4. Ligation and isolation of pBM11/PAD/nVGf α
- D. Preparation of pBM11/PAD/PF4

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Example VII.

Expression of the Polypeptide of Interest
as a Fusion Protein
with the Alkaline Phosphatase Signal Sequence

- A. Preparation of pBM11/PAK/nVGf α
- 30 B. Preparation of pBM11/PAK/EGF
- C. Preparation of TacPak/EGF

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Example VIII.

Expression of the Polypeptide of Interest
as a Fusion Protein
with the Alkaline Phosphatase Signal Sequence
Using an Expression Cassette
5 Comprising a Transcriptional Termination Region

A. Preparation of pTCPt/EGF

1. Preparation of the 420bp HindIII(blunt)-BamHI
fragment of TacPak/EGF
2. Preparation of the 2.8kb EcoRI(blunt)-BamHI
10 fragment of pBM16t/NDP/VGFa
3. Ligation and Isolation of pTCPt/EGF

B. Preparation of pTCPt/nVGFa

1. Preparation of the 350bp PvuI-BamHI fragment of
pBM11/PAK/nVGFa
2. Preparation of the 2.8kb PvuI-BamHI fragment of
15 pTCPt/EGF
3. Ligation and Isolation of pTCPt/nVGFa

C. Preparation of pTNPt/EGF

1. Preparation of 2.8kb PvuI-BamHI pTNPt
2. Preparation of 300bp PvuI-BamHI fragment of
20 pBM11/PAK/EGF
3. Ligation and Isolation of pTNPt/EGF

Example IX.Isolation of Recombinant Polypeptides25 A. Growth Factors Produced in pBM-Based Vectors Using the
PL Promoter and the ts CI Repressor

1. TGF and Modified TGF
 - a. N/TGF
- 30 2. Modified and Truncated VGF
 - a. PAD/nVGFa
 - b. NDP/VGFa
 - c. VGFa
 - d. NDP/VGFA

3. Chimeric TGF/VGF Hybrids
- a. N/TTV
 - b. NDP/TTV
 - c. NDP/VTV
 - d. NDP/TVV
- 5 B. Growth Factors Produced in Vectors Comprising the tac or lac Promoters
1. PAK/EGF
 2. PAK/nVGFa
- 10 C. Platelet Factor 4
1. N/PF4
 2. NDP/PF4
- D. Oncostatin M
1. NDP/Oncostatin M
 - 15 2. PAD/Oncostatin M

Example X.

Biological Activity of Recombinant Growth Factors
Prepared in Prokaryotic Cells

- 20 A. EGF Receptor Binding
1. Receptor Binding of Chimeric Peptides
- B. Mitogenic Activity
- C. Wound Healing
- 25 1. Mid-Dermal Injuries
2. Mid-Dermal Donor-Graft Injuries

Example XI.

30 Biological Activity of Recombinant Platelet Factor 4
Prepared in Prokaryotic Cells

- A. Inhibition of DNA Synthesis
- B. Inhibition of Growth of Tumors in Nude Mice

Example XII.

Biological Activity of Recombinant Oncostatin M
Prepared in Prokaryotic Cells

- 5 A. Physicochemical Characterization
 1. SDS-PAGE
- B. Growth-Inhibitory Activity of Recombinant Oncostatin M
- C. Receptor Binding Activity of Recombinant Oncostatin M

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Biological Deposits

The following expression plasmids, all transformed into E. coli HB101, were deposited on the indicated date with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 and have the identification and ATCC Designations given below:

	<u>IDENTIFICATION</u>	<u>ATCC DESIGNATION</u>	<u>DATE OF DEPOSIT</u>
10	pBM11	67366	
	pBM14	67367	
	pBM11/PA/VGF	67417	June 3, 1987
	pBM11/DP/VGFa	67418	June 3, 1987
15	pBM11/PA/EGF	67419	June 3, 1987
	pBM11/M5	67436	
	pBM11/C2	67437	
	pBM11/NDP/EGF	67547	October 23, 1987

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Methods

General cloning techniques were used as described in Maniatis et al., 1982 "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory, CSH, New York. All DNA-modifying enzymes were obtained from commercial suppliers. They were used according to the manufacturers instructions. Materials and apparatus for DNA purification and separation were used according to instructions from the supplier.

30

Example I.Activity Assays35 A. Mitogenic Assay

The mitogenesis assays were performed as follows: Diploid human fibroblasts obtained from explants

of newborn foreskin were seeded at a density of 3×10^4 cells/well (96-well plates, Nunclon, Roskilde, Denmark) and were grown to confluency in Dulbecco's modified Eagle's medium (GIBCO)/10% newborn calf serum. Cultures were then placed in medium containing 0.2% newborn calf serum, and two days later various concentrations of the growth factor to be tested were added. After 8 hrs, cultures were labeled with 5-[^{125}I]iodo-2'-deoxyuridine (Amersham, 10 $\mu\text{Ci/ml}$, 5 Ci/mg; 1 Ci = 37 GBq), and the amount of isotope incorporated into TCA insoluble material was determined as described (Twardzik et al., Proc. Natl. Acad. Sci. USA (1985) 182:5300-5304).

B. Soft Agar Colony Growth Stimulation Assay

A 0.5 ml base layer of 0.5% agar (Agar, Noble; Difco Laboratories, Detroit, Michigan) in growth medium was added to 24-well Costar tissue culture plates. One-half ml 0.3% agar in growth medium containing 1 to 1.5×10^4 cells/ml NRK cells or other cell line of interest and various concentrations of the factor to be tested was overlaid on the base layer of agar. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and refed after 7 days by addition of 0.5 ml of 0.3% agar in growth medium and containing the same concentration of the factor to be tested. Colonies were counted unfixed and unstained. The number of colonies with greater than 6 cells were scored.

C. EGF Receptor Binding Inhibition Assay

The radioreceptor assays were performed as follows: The binding of ^{125}I -labeled growth factor to its receptor on monolayers of a target cells was modified from the procedure described by Cohen and Carpenter, Proc. Natl. Acad. Sci. USA (1975) 72:1317-1321. Cells (1×10^3 per well) were fixed on 24-well plates (Linbro, Flow Laboratories) with 10% formalin in phosphate-buffered saline prior to assay. Formalin-fixed cells do not slough

off plates as easily as do unfixed cells, and replicate values were thus more consistent. Growth factor concentrations are expressed as ng equivalents of the native growth factor/ml i.e., the amount required to produce an inhibition of ^{125}I growth factor binding equivalent to that produced by a known amount of native growth factor.

D. Wound Healing

10 1. Mid-dermal Thermal Injuries

Mid-dermal thermal injuries were made on the dorsal thorax of anesthetized female Yorkshire pigs (30 lbs) whose backs had been shaved and depilatted with commercial hair remover cream. A brass template (3x3 cm, 147 gm) was equilibrated in a 70°C water bath and placed in firm contact with the skin for exactly 10 seconds. The resulting blister was then removed. Five mid-dermal burns were placed on each side of the spine and were separated from each other by approximately one inch. Burns were treated twice a day with approximately 3 ml of vehicle cream (Silvadene®) alone or containing growth factor or were untreated. After 9 days or 10 days of treatment, the pigs were anesthetized and eschar was removed from the burns. Biopsies were taken of each burn from re-epithelialized areas.

2. Mid-dermal Donor Graft Injuries

A 5-month-old 20.5 kg micropig was anesthetized with 20 mg/kg ketamine and 2 mg/kg Rompum. The dorsal thorax was shaved, prepped with betadine and thoroughly rinsed with saline. A series of 6 5x5 cm donor sites were made on each side of the dorsal thorax with a Padgett dermatome at 650/1000 inch by taking two swipes at 30/1000 inch. Topical therapy included 1 ml of saline in 20 gm of Silvadene® distributed evenly between the six wounds on the left side. The right side was treated with 1 ml of the growth factor to be tested, in 20 gm of Silvadene® divided evenly between the six wounds. All wounds were

covered with a large burn dressing, chux, ace wrap and
gerkin. The animal was anesthetized as described above on
post-operative days 1, 2, 3, 4, 7, 8, 9, 10 and 11. The
dressings were removed. The wounds were gently wiped with
5 betadine and thoroughly rinsed with saline. The
appropriate agent was applied and the wounds were
redressed as described above.

E. Inhibition of DNA synthesis

10 On day 2 in the morning A549 cells (human lung
carcinoma) in Nunc 96-well plates (Kamstrupvej 90. DK-
4,000, Roskilde, Denmark) were set up. These cells
were passaged when there were fewer than 30. Into all
but the peripheral wells was introduced 4×10^3 cells/
15 50 μ l/well (9×10^4 cells/ml assay medium (DMEM) with
10% FCS, P/S, glutamine). The peripheral wells re-
ceived 50 μ l PBS and the entire plate was incubated at
37°C. In the afternoon, the test compounds were resus-
pended in assay medium. All compounds were tested in
20 triplicate. Into each test well was delivered 50 μ l of
test compound in assay medium, while control wells re-
ceived 50 μ l assay medium alone. Each plate was then
incubated at 37°C for 3 days. On day 4, into each well
50 μ l of a solution of ^{125}I -iodo-2'-deoxyuridine (4 Ci/
25 mg to 0.5 mCi/ml) (1 μ l isotope/ml assay medium) was
added and the plates incubated at 37°C overnight. On
day 5, the medium was aspirated from the wells, and the
wells washed 1X with PBS. One hundred microliters of
methanol were added for 10 min at room temperature.
30 The methanol was aspirated and 200 μ l of 1 M sodium
hydroxide were added to each well. The plate was in-
cubated for 30 min at 37°C, and the sodium hydroxide
removed with Titertek plugs (Flow Labs). The plugs
were then counted in a gamma counter.

F. Inhibition of Tumor Growth in Nude Mice

Male nude mice (Balb/c-nu+/nu+) were supplied by the Fred Hutchinson Cancer Research Center, Seattle, WA. At 12 weeks of age, mice were given injections (s.c. in the neck region with approximately 1.3×10^6 human lung carcinoma cells (A549) in a volume of 0.2 ml of phosphate-buffered saline. Palpable tumors (approx. 10 mm³) usually developed in 20 days. Each group contained 5 animals. Animals were injected every two or three days at the tumor site with 0.1 ml of PBS (control group) or test sample (1.2 µg/injection) resuspended in 0.1 ml of PBS. Day one post-treatment corresponds to the first day animals were injected at the tumor site with test compounds. Tumor size was measured before subsequent injection on the days indicated and represents the average size of tumor in each animal in the group.

Example II.

20 Construction of Cloning and Expression Plasmids

A. Plasmid pBM11

Plasmid pBM11 contains the nucleotide sequence coding for the first 33 N-terminal amino acids of the bacteriophage lambda N protein. It also contains the neomycin resistance gene as a selective marker and a unique BamHI site for cloning of a foreign gene downstream from the lambda PL promotor. pBM11 was constructed as follows.

30 1. Construction of pBM4

A 2.4 kb DNA fragment containing the gene sequences coding for bacteriophage lambda cI was isolated by digesting (cleaving) 120 µg of lambda cI857S7 DNA (New England BioLabs) with 170 units of Bgl II (BRL) restriction enzyme by incubating the mixture for 2 hr. at 37°C. The digestion mixture was subjected to electrophoresis on a 1% preparative agarose gel

using techniques which are conventional in recombinant DNA technology. The 2.4 kb fragment (base pair 35771 to 38103; the numbering is based on Daniel, et al., pg. 519 in Lambda II, ed. by Hendrix, Roberts, Stahl, and Weisber,) was excised from the gel and subjected to electroelution at 100 V for 4 hr. at room temperature. The resulting eluate was recovered, concentrated, and extracted 3 times with an equal volume of phenol:chloroform (1:1). DNA was recovered from the aqueous phase by ethanol precipitation in the presence of 1/10 volume of 3M sodium acetate, pH 5.2. The recovery of the fragment was analyzed by agarose gel electrophoresis

Plasmid pPL-lambda (5ug, Pharmacia) (a pBR322 derivative containing the lambda leftward promoter P_L , the lambda N gene and the termination site for transcription of N (T_L) and 2 BamHI sites) was digested with 5 units of BamHI by incubating the mixture for 20 min. at 37°C. The digested DNA was separated by agarose-electrophoresis and the full-length DNA was recovered by electroelution using techniques which are conventional in the art.

The linearized p P_L -Lambda was treated with alkaline phosphatase to remove the 5' phosphates and ligated to the 2.4 kb BglII fragment of cI857S7 (3 μ l, about 250 ng), in the presence of ligase buffer (500mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 200 mM DTT, 10 mM ATP), and T4 DNA ligase (BRL). The resulting reaction mixture was incubated at 12°C for about 15 hr then used directly for transformation into competent E. coli HB101 cells.

E. coli cells were made competent by a modification of a procedure described by Hanahan, J. Mol. Biol., (1983) 166: 557-580. A saturated culture of the HB101 cells was diluted 1:200 in Luria Broth supplemented with 20 mM $MgCl_2$ and incubated at 37°C in a gyratory water bath until the culture reached an

optical density (OD) nm A₅₅₀, of 0.3. A 20 ml portion of the culture was harvested by centrifugation at 4°C. The resulting pellet was resuspended in 4 ml of ice cold 50 mM MnCl₂/20 mM potassium acetate, pH 6.0 and kept on ice for 15 min. The cells were centrifuged and the pellet was resuspended in 1.4 ml of a solution of 10 mM potassium methanesulfonate, pH 6.2, 100 mM of potassium chloride, 45 mM MnCl₂ .4H₂O, 10mM CaCl₂, 3 mM hexamine CoCl₃, 100 µl DMSO, and 100 µl 1M DTT.

Three hundred µl of the treated cells were added to 4 µl and 6 µl of the ligation mixture. The treated cells were placed on ice for 30 min., incubated at 42°C for 90 sec., and then placed on ice again for 90 sec. The cells were then plated on L-Broth agar plates supplemented with 100 µg/ml of ampicillin. As there is no rapid and convenient method to screen for bacterial transformants containing the insert, the transformants were screened by isolating the various DNA by the rapid plasmid DNA isolation procedure of Holmes and Quigley Anal. Biochem., (1981) 114, 193-198. Plasmid DNA preparations from recombinant bacteria which migrate with the "correct" size were further analyzed by restriction map analysis to determine the orientation of the insert.

2. Construction of pBM8

Plasmid pBR322 (5µg) was digested with PstI (3ul, New England BioLabs) followed by 3 successive extractions using a 1:1 (v:v) phenol:chloroform solution followed by 2 ether extractions. The DNA was precipitated completeness of digestion was analyzed by electrophoresis on 0.8% agarose gel. The PstI digested pBR322 (10 µl) was treated with T4 polymerase (1 µl, BRL) to convert the 3' protruding ends to blunt ends. A HindIII site was introduced at the PstI site of pBR322 by ligation of a synthetic phosphorylated HindIII* linker (5'-d[CAAGCTTG]) (Pharmacia). The resulting DNA was digested with excess HindIII. Two

DNA fragments, a 3.58 Kb fragment and a 782 bp fragment were obtained. The 3.58 Kb fragment was isolated, ligated, and transformed into competent E. coli HB101. The transformants were analyzed as described above by sizing the HindIII digested DNA on agarose gels.

3. Construction of pBM9

Plasmid pBM8 was digested with HindIII and BamHI to yield 2 fragments of DNA. The larger fragment, 3.23 Kb, was isolated by agarose gel electrophoresis and recovered by electroelution. Plasmid pNeo (Pharmacia) was digested with HindIII and BamHI to yield a 1.5 Kb fragment containing the gene sequences coding for neomycin resistance. The 1.5 Kb fragment was isolated and purified, then ligated to the 3.23 Kb fragment from pBM8. The resulting plasmid, pBM9, was transformed into competent E. coli HB101 and the transformants were screened as described previously.

4. Construction of pBM10

Plasmid pBM9 was digested with NdeI (New England BioLabs). The 5' protruding end was converted to blunt end using E. coli DNA Polymerase "Klenow" fragment and ligated to a synthetic phosphorylated EcoRI linker (5'-d[GGAATTCC]-3') at the filled in NdeI site. The resulting plasmid, pBM10, an NdeI site and gained an EcoRI site pBM10 was transformed into competent E. coli HB101 and the transformants were screened as described previously.

5. Final Construction of pBM11

Plasmid pBM10 was digested to completion with EcoRI and BamHI. The resulting 2.8 Kb fragment containing the neomycin gene and origin of replication was isolated by agarose gel electrophoresis and recovered by electroelution. Plasmid pBM4 (described above) was digested to completion with EcoRI and BamHI. The 2.84 Kb fragment containing the DNA

sequences for the cI gene and lambda P_L promoter and the N gene ribosomal binding site was isolated and recovered. It was then ligated to the 2.84 Kb pBM4 fragment to form pBM11. Plasmid pBM11 transformed into competent E. coli HB101 and the transformants obtained were screened as described previously. The cells containing pBM11 were screened further by restriction enzyme analysis with BamHI.

10 B. Construction of pBM11M4

This plasmid was derived from pBM11 and allows a foreign gene to be cloned at a BamHI restriction site directly after the initiating methionine of the N gene. Plasmid pBM11/M4 also contains an NcoI site in the neomycin gene. It was constructed as follows:

15 1. Construction of pBM11/M

Plasmid pBM11 (20 ug) was cleaved with BamHI and PvuI. After complete digestion, the DNA was electrophoresed through an 0.8% agarose gel and the large fragment (5124 bp) was isolated and recovered by electroelution. A second sample of pBM11 (10 ug) was digested to completion with SphI and the 3' protruding ends converted to blunt ends with T4 DNA polymerase. 0.3 ug of each of the two fragments were mixed together with 37.5 pmol of the phosphorylated synthetic oligodeoxynucleotide (Pharmacia P-L Biochemical)

5' AGGAGAATTCATATGGATCCACAA 3'

30 containing the restriction sites EcoRI, NdeI and BamHI. The resultant plasmid was designated pBM11/M. The plasmid pBM11/M was heated at 100°C for 3 minutes, then sequentially cooled as follows: 30°C for 30 minutes, 4°C for 30 minutes, and 0°C for 10 minutes. 35 The reannealed DNA was subsequently treated with T4 DNA ligase and transformed into E. coli HB101. Neomycin-resistant transformants were screened for plasmids

containing the three new restriction sites encoded by the synthetic oligonucleotide.

2. Construction of pBM11/M1

Plasmid pBM11/M was digested to completion with BamHI then electrophoresed through a 0.7% agarose gel. The large fragment (5554 bp) was isolated and recovered by electroelution. The resultant plasmid, which lacks the 100 bp region of the lambda N gene downstream from the ribosomal binding the translation initiation site, was designated pBM11/M1. Plasmid pBM11/M1 was religated and transformed into competent E. coli HB101.

3. Construction of pBM11/M2

A first sample of plasmid pBM11 (0.3 ug) was cleaved with BamHI and PvuI. A second sample of plasmid pBM11 (0.3 ug) was cleaved with SphI and then treated with T4 polymerase. The two samples were combined with 3.75 pmol of the phosphorylated synthetic oligodeoxynucleotide (Pharmacia P-L Biochemical)

5' AGGAGAATCCAGATGGATCCACAA 3'

which contains only a BamHI. The mixture was heated and cooled as described previously, and treated with T4 DNA ligase and E. coli DNA polymerase "Klenow" fragment. The resultant plasmid, designated pBM11/M2, was transformed into competent E. coli HB101. Neomycin-resistant colonies were screened for plasmids containing the new BamHI site encoded by the synthetic oligodeoxynucleotide.

4. Construction of pBM11/M3

Plasmid pBM11/M2 was digested to completion with BamHI. The cleaved DNA was electrophoresed through a 0.7% agarose gel and the large fragment (5554 bp) isolated and recovered by electroelution. The DNA

religated and designated pBM11/M2 transformed into competent E. coli HB101.

5. Final Construction of pBM11/M4

Plasmid pBM11/M1 (20 ug) was digested to completion with EcoRI and BamHI, then electrophoresed through a 0.7% agarose gel. The large fragment (5544 bp) was isolated and recovered by electroelution. This DNA was ligated to the following pair of phosphorylated synthetic oligodeoxynucleotides (200pmol each):

5' AATTCCTCATGGGG 3'

and

3' GGGTACCCCTAG 5'

which contains a NcoI site. The mixture was heated to 65°C and allowed to cool to 25°C in order to anneal. The resultant plasmid designated pBM11/M4, was transformed into competent E. coli HB101. Neomycin resistant colonies were screened for plasmids containing a new NcoI site.

C. Construction of pBM11/M5

Plasmid pBM11/M5 was derived from pBM11. An NcoI site present in the neomycin resistant gene has been removed by self directed mutagenesis. Plasmid pBM11/M5 is identical to pBM11/M4 except that the NcoI site in the neomycin gene has been removed by site-specific mutagenesis. Cloning of foreign genes into pBM11/M5 therefore does not require partial digestion of the vector with NcoI.

D. Construction of pBM11/C Series

A pBM11 plasmid containing the E. coli consensus ribosomal binding site was produced and designated "pBM11/C." Plasmid pBM11, 30 ug, was digested with 80 ug PvuI and 192 units BamHI. The

resulting two fragments (5.1kb and 0.53kb) were separated by gel electrophoresis and isolated by electroelution. The 0.53kb fragment was then digested with HaeIII. The resulting fragments were separated by gel electrophoresis and the 324bp fragment was isolated by electroelution. The construct pBM11C, the 5.1kb, the 324bp fragments isolated from plasmid pBM11 and a phosphorylated chemically synthesized linker,

10 5' GTAAGGAGGTTTAATATTATG 3'
 3' CATTCCTCCAAATTATAATACCTAG 5',

were ligated together. The plasmid thus constructed is termed pBM11/C. Plasmid pBM11/C has restriction site BamHI as the cloning site as well as a unique SspI restriction site within the spacer region of the ribosomal binding site. It has been reported that spacer length affects the efficiency of protein translation. The presence of the SspI site allows the length of the spacer region to be changed and also allows for the insertion of other cloning sites for other genes to be expressed.

Modification of plasmid pBM11/C has also been performed. pBM11/C, 10 ug, was digested with restriction enzyme SspI, 45U, and the 5'phosphate was removed by phosphatase. This DNA was ligated to a phosphorylated synthetic NcoI linker CCATGG. The resulting plasmid, termed pBM11C/1, contains 2 NcoI sites, one in the neomycin gene and one for the cloning of the foreign gene(s). To avoid performing NcoI partial digestion of pBM11/C1 in the cloning of foreign gene(s), the NcoI restriction site in the neomycin gene was removed by site-specific mutagenesis using techniques described previously. This plasmid is termed pBM11/C2.

E. Construction of pBM11/NDP

Plasmid pBM11/NDP, derived from plasmid pBM11, contains the nucleotide sequences coding for the first 32 amino acids of the lambda N protein followed by nucleotide sequences coding for the acid cleavable aspartic acid-proline dipeptide. The plasmid contains an NcoI site and a ClaI site for cloning a foreign gene downstream of the P_L promoter.

10 F. Plasmid pBM16t

This plasmid is identical to pBM11/NDP except that it lacks the NcoI site in the neomycin gene as described above (Example II.F.) and it contains the transcription terminator as described for plasmid pTNPt (see Example (II.N. for terminator sequence).

G. Construction of Plasmid pBM16/NDP

Plasmid pBM11/NDP/VGfa (see Example V.A.1. below) was digested with NcoI and BamHI which removes the synthetic gene TVV from the pBM16/DP plasmid fragment. The pBM16/NDP NcoI-BamHI 5.5 kb plasmid fragment was then gel purified. The NcoI site in this fragment is positioned downstream of the nucleotide sequences coding for the first 32 amino acids of the N-gene and directly after the sequences coding for the acid labile dipeptide Asp-Pro.

H. Construction of pBM11/PAD (also called pBM11M3PAD)

This plasmid is derived from pBM11/M3 and allows a foreign gene to be cloned at a HindIII, SmaI or BamHI site downstream from a modified alkaline phosphatase signal sequence. Synthetic oligonucleotides were designed to allow insertion of DNA coding for a modified alkaline phosphatase signal peptide and a linker region with 3 cloning sites (HindIII, SmaI and BamHI) into the pBM11 expression vector downstream from the P_L promoter and N gene ribosomal binding site. The

nucleotide sequence was optimized to be as similar as possible to the nucleotide sequence of the amino terminus of the lambda N gene as the lambda N gene sequence has evolved with that of its ribosomal binding site for efficient ribosome initiation and translation.

The sequences of the oligonucleotides coding for the signal sequence are shown below.

10

PA1 5' GATCAATCTACAATCGCCCTCGCACTTCTCCCACTGCTGTTCACTCC
AGTGACAAAAGCTTCCCGGG 3'

PA2 5' GATCCCGGGAAGCTTTTGTCACTGGAGTGAACAGCAGTGGGAGAAGT
CGGAGGGCGATTGTAGATT 3'

15

Oligonucleotides A1 and A2 were synthesized on a Applied Biosystems Oligonucleotide Synthesizer and purified on an acrylamide gel. The oligonucleotides were phosphorylated at the 5' end using T4 polynucleotide kinase and then annealed to each other yielding a double stranded 0.067kb DNA fragment with a BamHI overhang at each end.

pBM11/PAD was constructed as follows:

Plasmid pBM11/M3 (20ug) was digested with 30 units of BamHI to linearize the plasmid directly after the N gene ribosomal binding site and the ATG codon for the initiating methionine. The 5' phosphates were removed by digestion with calf intestinal alkaline phosphatase.

30

The 0.067kb PA oligonucleotide fragment was ligated to the linearized pBM11/M3 plasmid and the resulting DNA was used to transform competent E. coli HB101. The transformants were screened by nucleotide sequencing, and a correct construct was isolated.

35

I. Construction of pBM14

1. Construction of pBM12

Plasmid pBR322 was digested with restriction enzyme PstI. The resulting 3' protruding end was converted to a blunt end by treating the digestion product with T4 DNA polymerase according to conventional techniques. Synthetic BglII linker (5'-d[CAGATCTG]) was phosphorylated and ligated onto the blunt end DNA and used to transform competent E. coli HB101. Plasmid DNA preparations were prepared from tetracycline-resistant transformants using conventional techniques digested with PstI and BglII to screen for loss of the PstI restriction site and addition of the BglII restriction site.

2. Construction of pBM13

Plasmid pBM4, obtained as described above, was digested with HpaI. The 3' protruding end was converted to a blunt end and the phosphorylated BglII linker added to the blunt end. The ligation reaction mixture was used to transform competent E. coli HB101.

3. Final Construction of pBM14

Plasmid pBM12 was digested with BglII and EcoRI. The resulting 3.6 Kb fragment containing the tetracycline gene and origin of replication was isolated and recovered. Plasmid pBM13 was digested with BglII and EcoRI and the resulting 2.8 Kb fragment containing the bacteriophage lambda cI857 DNA sequences and the lambda P_L and the N ribosomal binding site was isolated and recovered. The 3.6 Kb fragment from pBM12 and 2.8 Kb fragment from pBM13 were ligated to form plasmid pBM14. The resulting ligation mixture was transformed into E. coli HB101 and the resulting transformants screened for pBM14. The putative pBM14 was analyzed further by restriction digest using conventional techniques.

J. Plasmid pLEBam

Plasmid pLEBam was used to clone synthetic oligonucleotide fragments because of its convenient BssHII and BamHI restriction sites. A plasmid with NcoI and EamHI restriction sites such as pBM11 or pBM11/NDP (described below) can be used for cloning the synthetic nucleotide fragments.

K. Plasmid plac/cro- β gal.

The controlling elements of the vector plac/cro- β gal consist of the operator-promoter region of E. coli lactose (lac) operon, as well as the ribosome-binding sites of lac and cro. This vector is derived from plasmids pTR213 (Roberts *et al.*, Proc. Natl. Acad. Sci. USA (1978) 76:760) and pLG300 (Guarante *et al.*, Cell (1980) 20:543).

Plasmid plac/cro- β gal was constructed by ligating a 0.96 kb PstI-BglII fragment from pTR213 and a 5.54 kb PstI-BamHI fragment from pLG300 in the presence of the oligonucleotide linker which had been digested with BamHI and BglII:

AAAGATCTCAGGCCTCGAGGATCC
TTTCTAGAGTCCGGATCTCCTAGG

This linker served the following purposes:
(1) to regenerate the BglII and BamHI sites from the parental plasmids, (2) to provide additional sites for the insertion of foreign DNA, and, (3) to allow the inserted DNA to be in the correct translational reading frames with respect to the cro 5'-gal coding sequence.

L. Plasmid ptac/cro- β gal

Plasmid ptac/cro- β gal allows a foreign gene to be cloned downstream of the N-terminal 21 amino acids of the bacterial Cro protein. It was constructed by inserting a 0.87 kb RsaI fragment of the plac/cro- β gal

plasmid into pDR540 (Pharmacia) at the BamHI site, which was previously converted to blunt ends by the action of Klenow enzyme. The orientation of the inserted DNA was such that the ribosomal binding site and the coding sequence of Cro were located downstream from the ribosomal binding site of lac. The resulting plasmid, ptac/cro, contained ribosomal binding sites of both lac and cro, and the N-terminal coding sequences of Cro.

The second step in the construction of ptac/cro- β gal was to ligate the 1.16 kb and the 5.54 kb PstI-BamHI fragments from ptac/cro and pLG400 plasmids, respectively. Expression vector ptac/cro- β gal is thus similar to plac/cro- β gal, with the exception that the promoter of ptac/cro- β gal consists of the -35 region from the promoter of the tryptophan operon and the Pribnow box (-10 region) of the lac operon. This hybrid promoter allows a higher level of expression than plac/cro- β gal.

M. TacPak

For preparation see Example VII c.

N. Plasmid pTCPt

This plasmid is designed to have the tac promoter elements and utilize the cro SD to express the gene of interest behind the alkaline phosphatase signal sequence. An example of the construction of this plasmid is given below in the construction of pTCPt/EGF.

O. Construction of pTNPt ([trp-35]17bp[lac-10][nSD]8bp[ATG]/alkaline phosphatase signal/linker/trans. term.-NEO)

This plasmid is designed to have the tac promoter elements and utilize the N-gene SD to express a given gene behind the alkaline phosphatase signal

sequence. It has a pBR322 background with the Neomycin resistance gene. Plasmid pTNPt was constructed as follows:

1. Preparation of the 2.8kb EcoRI-BamHI fragment of pBM16t/VGFa lacking the HindIII site

5 Plasmid pBM16t/VGFa was digested with EcoRI and BamHI and the 2.8kb fragment was isolated. The 2.8kb fragment was ligated to an EcoRI-BamHI linker and a correct construct was isolated by restriction
10 analysis and is referred to as Intermediate I.

The unique HindIII site near the Neomycin resistance gene was removed from the Intermediate I plasmid by digestion with HindIII, creating blunt ends using Klenow fragment, and religating. This resulted
15 in Intermediate II plasmid which lacked the HindIII site.

The 2.8kb EcoRI-BamHI fragment of pBM16t/VGFa lacking the HindIII site was isolated by digesting pIntermediate II with EcoRI and BamHI. The resulting
20 2.8kb fragment was isolated by agarose gel electrophoresis.

2. Preparation of the 150bp BamHI-BsmI fragment of pBM11/PAK

Plasmid pBM11/PAK is identical to
25 pBM11/PAK/EGF except that it contains a linker region with HindIII, SmaI and BamHI sites downstream of the alkaline phosphatase signal sequence instead of the EGF gene. pBM11/PAK was digested with BsmI and BamHI and the 150bp fragment containing the N-gene SD, the
30 alkaline phosphatase signal sequence and the linker region was isolated.

3. Preparation of oligonucleotides TacA+ and TacA-

Oligonucleotides TacA+ and TacA- were
35 synthesized on an Applied Biosystems Oligonucleotide synthesizer and were designed to have an EcoRI overhang at the 5' end with the trp-35 consensus sequence

separated from the lac-10 consensus sequence by 17 nucleotides within which was positioned a SstI site. The sequence also contained the 5' end of the lac mRNA, the lac repressor binding site and a BsmI overhang.

5

TacA+ 5' AATTACTCCCCATCCCCCTGTTGACAATTAATCATCGAGCTC
GTATAATGTGTGGAATTGTGAGCGGATAACAATTTACACAG 3'

10

TacA- 5' GTGTGAAATTGTTATCCGCTCACAATTCCACACATTATA
CGAGCTCGATGATTAATTGTCAACAGGGGGATGGGGAGT 3'

4. Ligation and Isolation of pTNPt

The 2.8kb EcoRI-BamHI fragment, the 150bp BsmI-BamHI fragment and oligonucleotides TacA+ and TacA- were ligated together using DNA ligase and the DNA was used to transform competent JM109(lacIq). A correct construct was isolated by restriction analysis and DNA sequencing.

20

(EcoRI site of pBM11)

GAATTACTCCCCATCC

25

trp-35 (17bp) SstI lac-10
CCCTG [TTGACA] ATTAATCATCGAGCTCG (TATAATG)

30

5' lac mRNA-> BsmI n mRNA->
TGTGG/AATTGTGTGAGCGGATAACAATTTACACAGCATTCAAAGCAGAAGGCT

TTGGGGTGTGTGATACGAAACGAAGCATTGGCCGTAAGTGCGATTCCGGATTAGC

TGCCAATGTGCCAATCGCGGGGGGTTTTCGTTTCAGGACTACAACCTGCCACACACC

35

^{mSD} (8bp) ^{Signal Sequence ->} ^{PvuI}
 ACCAAAGCTAACTGAC {AGGA} GAATCCAG ATGAAACAATCTACGATCGCCC
 M K Q S T I A L

5 ^{HindIII} ^{SmaI} ^{BamHI}
 TCGCACTTCTCCCACTGCTGTTCAGTCCAGTGACAAAAGCTTCCCGGGATCCGTG
 A L L P L L F T P V T K

10 ^{Trans. Term.} ^(BamHI site of pBM11)
 ACTAATTGGGGACCCTAGAGGTCCCCTTTTTTATTTTAAAACGATCC

Example III.

Preparation of Genes of Interest

15

A. Synthetic Growth Factor Genes

Synthetic growth factor genes were designed which use host cell codons optimized for high levels of expression. In addition, several convenient restriction sites were designed into the synthetic genes. When possible, the new restriction sites left the amino acid sequence of the growth factor gene unaltered, however, in some cases incorporation of the new restriction site yielded an altered amino acid sequence. These sites roughly divide the synthetic genes into thirds yielding N-terminal, middle and C-terminal domains.

The natural VGF gene product contains an extreme N-terminal domain which has no counterpart in mature TGF. VGF fragments lacking this domain are referred to as truncated. The restriction sites were used for initial construction of the final genes from partial synthetic oligonucleotide fragments extending from one restriction site to another. The oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer and were purified on an acrylamide gel. The oligonucleotides were phosphorylated at

the 5' end using T4 polynucleotide kinase and each oligonucleotide was then annealed to its complement.

1. TGF Synthetic Oligonucleotides

5 a. Human TGF N-terminal domain

TGF ->

BssHIINcoI

10 M V V S H F N D C P D S H T Q F
5' CGCGCCATGGTTGTTTCTCACTTTAACGACTGCCCGGACTCTCATACTCAGT
3' GGTACCAACAAAGAGTGAAATTGCTGACGGGCCTGAGAGTATGAGTCA

KpnI

15 C F H G T
TTTGCTTTTCATGGTAC 3' TGF104
AAACGAAAGTAC 5' TGF103

b. Modified human TGF middle domain with the human sequence QEDK being altered to QEEK, the sequence found in rat TGF

20

KpnI

SphI

25 C R F L V Q E E K P A C
5' CTGCCGTTTTCTGGTTCAGGAAGAAAAACCGGCATG 3' TGF101
3' CATGGACGGCAAAGACCAAGTCCTTCTTTTGGCC 5' TGF102

c. Human TGF C-terminal domain:

30

SphI

V C H S G Y V G A R C E H A D L
5' CGTTTGCCATTCTGGCTACGTTGGCGCACGTTGCGAACACGCTGACCT
3' GTACGCAAACGGTAAGACCGATGCAACCGCGTGCAACGCTTGTGCGACTGGA

35

BamHI

L A Ter
GCTGGCTTAAG 3' TGF205
CGACCGAATTCCTAG 5' TGF206

2. VGf Synthetic Oligonucleotidesa. VGf extreme N-terminal domain

5

HindIII

E D S G N A I E T T S P E I T N A
 5' AGCTGACTCTGGTAACGCTATCGAACTACTTCTCCGGAAATCACTAACGCT
 3' CTGAGACCATTGCCGATAGCTTTGATGAAGAGGCCTTTAGTGATTGCGA

10

T T
 ACTACT 3' VGF105
 TGATGA 5' VGF106

15

b. Modified VGf N-terminal domain including
 Asp-Pro cleavage site, with the sequence
HGT replacing the natural sequence HGD

20

BamHI

I D P M D I P A I R L C G P E G D
 5' GATCGATCCCATGGACATCCCGGCTATCCGTCTGTGCGGCCCGGAAGGCGAC
 3' CTAGGGTACCTGTAGGGCCGATAGGCAGACACGCCGGGCCTTCCGCTG

25

KpnI

G Y C L H G T
 GGCTACTGCCTGCATGGTAC 3' VGF104a
 CCGATGACGGACGTAC 5' VGF103a

30

c. Modified VGf middle domain having the
 sequence GYAC replacing the natural
sequence GMYC

35

KpnISphI

T C I H A R D I D G Y A C
 5' CTGCATCCATGCACGTGACATCGACGGCTACGCATG 3' VGF101a
 3' CATGGACGTAGGTACGTGACTGTAGCTGCCGATGC 5' VGF102a

gel. The oligonucleotides were phosphorylated at the 5' end using T4 polynucleotide kinase. Each oligonucleotide was annealed to its complement.

5

NcoIEcoRI
 M N S D S E C P L S H D G
 5' CATGAATTCTGACTCTGAATGCCCCTGTCTCATGACGGC
 3' TTAAGACTGAGACTTACGGGCGACAGAGTACTGCCG

10

Y
 TAC 3' EGF1A
 ATGACGGAC 5' EGF2A

15

NsiI
 C L H D G V C M Y I E A L D K Y A
 5' TGCCTGCATGACGGCGTATGCATGTACATCGAAGCTCTGGACAAGTACG
 3' GTACTGCCGCATACGTACATGTAGCTTCGAGACCTGTTTCATGC

20

SphI
 C
 CATG 3' EGF1B
 5' EGF2B

25

SphI
 N C V V G Y I G E R C Q Y R D
 5' CAACTGCGTTGTTGGCTACATCGGCGAACGTTGCCAGTACCGTGAC
 3' GTACGTTGACGCAACAACCGATGTAGCCGCTTGCAACGGTCATGGCACTG

BamHI
 L K W W E L R *
 CTGAAATGGTGGGAACCTGCGTTAAG 3' EGF3
 GACTTTACCACCCTTGACGCAATTCCTAG 5' EGF4

30

4. Assembly of Growth Factor Genes

a. Preparation of Plasmid pLEBam/TTV

35

The synthetic chimeric growth factor, denoted TTV or (TGF/TGF/VGF) was assembled in the cloning vector pLEBam. This hybrid growth factor contained the amino acid sequence of human TGF in the amino terminal two-thirds of the gene with the exception of the se-

quence QEEK which was altered from the natural human sequence QEDK. The carboxy terminus was derived from the amino acid sequence of VGF and terminated with the sequence YQR TMR upstream of the natural sequence PNT. Plasmid pLEBam was digested with BssHII and BamHI. BssHII-BamHI pLEBam was then ligated to oligonucleotides TGF101, 102, 103, and 104, and VGF1, 2, 3, and 4 using DNA ligase and the resulting plasmids used to transform competent HB101. The transformants were selected on ampicillin and screened by restriction analysis using EcoRI, NcoI and BamHI and by nucleotide sequencing using the Maxam-Gilbert protocol. A correct construction was isolated and denoted pLEBam/TTV.

-15

TGF →
NcoI
 CCATGGTTGTTTCTCACTTTAACGACTGCCCGGACTCTCATACTCAGTTTGGCTT
 M V V S H F N D C P D S H T Q F C F

20

VGF →
KpnI SphI
 TCATGGTACCTGCCGTTTCTGGTTCAGGAAGAAAAACCGGCATGCGTTTGCTCT
 H G T C R F L V Q E E K P A C V C S

25

EcoRI
 CATGGCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGGTCGACTACCAGCGT
 H G Y T G I R C Q H V V L V D Y Q R

30

BamHI
 TAAGGATCC
 Ter

35

b. Preparation of Plasmid pLEBam/TVV

The synthetic chimeric growth factor, denoted TVV or (TGF/VGF/VGF) was assembled in the cloning vector pLEBam. This hybrid growth factor contained the amino acid sequence of human TGF in the N-terminal domain of the gene. The middle and C-terminal domains

are derived from the truncated VGF sequence and end with the sequence YQR upstream of the natural sequence PNT. In addition, the synthetic gene has the modification, GYACVC for GMYCRC.

5 (1) Preparation of a 4.3kb KpnI-SphI
fragment of pLEBam/TTV

Plasmid pLEBam/TTV was digested with KpnI and SphI and the 4.3kb KpnI-SphI fragment was gel purified. This digestion removes the middle TGF domain from the synthetic gene TTV in the cloning plasmid pLEBam.

(2) Ligation and isolation of pLEBam/TTV
 Oligonucleotides VGF101a and 102a were ligated to the 4.3kb KpnI-SphI fragment of pLEBam/TTV using DNA ligase and the resulting mixture was used to transform competent HB101. The transformants were selected on ampicillin and were screened by nucleotide sequencing using the Sanger-dideoxy method. A correct construct was isolated and denoted pLEBam/TTV.

20

TGF +

NcoI
 CCATGGTTGTTTCTCACTTTAACGACTGCCCGGACTCTCATACTCAGTTTTGCTT
 M V V S H F N D C P D S H T Q F C F

25

VGF +

KpnI SphI
 TCATGGTACCTGCATCCATGCACGTGACATCGACGGCTACGCATGCGTTTGCTCT
 H G T C I H A R D I D G Y A C V C S

30

EcoRI
 CATGGCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGCTCGACTACCAGCGT
 H G Y T G I R C Q H V V L V D Y Q R

35

BamHI
 TAAGGATCC
 Ter

B. Synthetic Platelet Factor 4 Gene

A synthetic platelet factor 4 gene was designed which uses bacterial codons optimized for high levels of expression. Single-stranded overlapping sequences were prepared, combined in an annealing medium and ligated to provide the complete gene with appropriate termini for insertion into an expression vector in reading phase to prepare a fused protein from which platelet factor 4 could be isolated. The resulting expression vector was called pHCPF4. The single-stranded segments were 5'-phosphorylated with T4 polynucleotide ligase and annealed by combining 200 pM of each segment in a 30 μ l reaction volume (30 mM ATP, 10 mM DTT, 10 mM MgCl₂, 1 μ g/ml spermidine, 100 mM Tris-HCl, pH 7.8 and T4 DNA ligase. The dsDNA was digested with BssHII and BamHI and purified on a 7% native polyacrylamide gel.

The following sequence was prepared:

20
3' GGTACCTTCGACTTCTTCTGCCTCTAGACGTCACG
5' CGCGCCATGGAAGCTGAAGAAGACGGAGATCTGCAGTGC
M E A E E D G D L Q C
|
NH₂

25
3' GACACGCATTTTTGATGAAGAGTCCATTCCGGAGCAGTG
5' CTGTGCGTAAAACTACTTCTCAGGTAAGGCCTCGTCAC
L C V K T T S Q V R P R H

30
3' TAGTGTAGTGAGCTCCATTAGTTTCGGCCGGGCGTCACGGGC
5' GGCATCACATCACTCGAGGTAATCAAAGCCGGCCCCGACCCG
I T S L E V U K A G P H C P

35
3' TGACGAGTCGACTAGCGCTGAGACTTTTTGCCAGCATTC
5' ACTGCTCAGCTGATCGCGACTCTGAAAAACGGTCGTAAG
T A Q L I A T L K N G R K

3' TAGACAGATCTGGACGTCCGAGGCGACATGTTTTTTTAG
5' ATCTGTCTAGACCTGCAGGCTCCGCTGTACAAAAAATC
I C L D L Q A P L Y L L I

3'TAGTTTTTTGACGACCTTAGAATTCCTAG
 5'ATCAAAAAACTGCTGGAATCTTAAG
 I K K L L E S ***

 |
 COOH

5

C. DNA Cloning of Oncostatin M

1. Preparation of cDNA Libraries

Poly(A)⁺ RNA obtained from U937 cells treated with media containing phorbol 12-myristate 13-acetate PMA (10 ng/ml) for 16, 36, and 52 hours was pooled and used for cDNA synthesis and cloning into a λ gt 10 vector, essentially as described by Huynh *et al.*, DNA Cloning Techniques: A Practical Approach, D. Glover (ed) (1984). Briefly, 10 μ g poly(A⁺) RNA was reverse transcribed in the presence of 50 pmol oligo dT. The second strand was synthesized using DNA polymerase I and the cDNA was treated with S1 nuclease to eliminate the hair pin loop. The cDNA was then dG tailed by treatment with terminal deoxy nucleotidyl transferase. The dG tailed cDNA was subsequently chromatographed on Biogel A-50 column to eliminate cDNA smaller than 300 bp. The sized dG tailed cDNA were ligated into EcoRI cut λ gt 10 in the presence of single stranded 16 nucleotide-long linker molecule comprising, from the 5' end, AATT followed by 12 deoxycytosine residues (Webb *et al.*, 1987). The ligated DNA was packaged in vitro (Grosveld *et al.*, Gene (1981) 13:227-237) and the phage was used to infect E. coli C60 Hfl⁺. This technique gave 3x10⁶ recombinants/ μ g cDNA. Nitrocellulose filter plaque lifts were done in duplicate and the filters were probed using long, best guess 35 to 50 nucleotide long probes. The oligonucleotide probes were derived from the peptide sequences obtained by automated repetitive Edman degradation. The purified Oncostatin M sequence was either derived from the N-

terminal of the protein or by sequencing the protease-generated lysine peptides.

Initial screening of the λ gt 10 library was done using a 50 mer oligonucleotide probe. The probe
5 was derived from the lysine peptide.

Peptide 1:

10 (K) A Q D L E R S G L N I E
3' TTC CCG GTC CTG GAC CTC GCC AGA CCG GAC TTG TAA CTC

 D L E K
CTG GAC CTC TT 5'

15 λ gt 10 clones showing positive reactivity to [32 P]-labeled above oligonucleotide were plaque purified. Eight clones were obtained. Southern blot analysis showed that the positively reacting cDNA inserts in the clones ranged between 600 bp to 2 Kb. Subsequently,
20 Southern blots were done using a 35 mer oligonucleotide (encoding amino acids 53-64) and a 41 mer oligonucleotide (encoding amino acids 22-35). Only one clone showed positive reactivity with all three radiolabeled oligonucleotide probes.

25 The cDNA insert of the λ gt 10 clone (λ OM) was found to be approximately 2.1 Kb. The cDNA insert flanked by EcoRI sites at 5' and 3' ends was subcloned in the EcoRI site of the polylinker region of the plasmid vector pEMBL18 (Dente et al., Nucleic Acid Res.
30 (1983) 11:1645-1655. The recombinant was termed pOncM46. Subsequently, additional cDNA clones were obtained by specific priming using oligonucleotides derived from the 5' coding region of the Oncostatin M gene and a genomic clone containing the entire gene was
35 isolated.

2. Restriction Site Mapping

A restriction map of the clone pOncM46 coding Oncostatin M protein was obtained by standard single or double digestions of the plasmid DNA. The coding
5 region has four PstI sites, a BglIII site and a SmaI site.

3. DNA Sequence of Oncostatin M

The entire nucleotide sequence of the cDNA clones was obtained and a consensus sequence was
10 determined as follows:

```
CGGGCCGGAGCACGGGCACCCAGCATGGGGGTACTGCTCACACAGAGGAC
      M G V L L T Q R T
15 GCTGCTCAGTCTGGTCCTTGCACTCCTGTTTCCAAGCATGGCGAGCATGG
   K K S K V K A K K F O S N A S M A
   CGGCTATAGGCAGCTGCTCGAAAGAGTACCGCGTGCTCCTTGGCCAGCTC
   A I G S C S K E Y R V L L G Q L
   CAGAAGCAGACAGATCTCATGCAGGACACCAGCAGACTGCTGGACCCCTA
20 Q K Q T D L M Q D T S R L L D P Y
   TATACGTATCCAAGGCCTGGATGTTCTAAACTGAGAGAGCACTGCAGGG
   I R I Q G L D V P K L R E H C R E
   AGCGCCCCGGGGCCTTCCCCAGTGAGGAGACCCTGAGGGGGCTGGGCAGG
   R P G A F P S E E T L R G L G R
25 CGGGGCTTCCTGCAGACCCTCAATGCCACACTGGGCTGCGTCCTGCACAG
   R G F L Q T L N A T L G C V L H R
   ACTGGCCGACTTAGAGCAGCGCCTCCCCAAGGCCCAGGATTTGGAGAGGT
   L A D L E Q R L P K A Q D L E R S
   CTGGGCTGAACATCGAGGACTTGGAGAAGCTGCAGATGGCGAGGCCGAAC
30 G L N I E D L E K L Q M A R P N
   ATCCTCGGGCTCAGGAACAACATCTACTGCATGGCCCAGCTGCTGGACAA
   I L G L R N N I Y C M A Q L L D N
   CTCAGACACGGCTGAGCCACGAAGGCTGGCCGGGGGGCCTCTCAGCCGC
   S D T A E P T K A G R G A S Q P P
35 CCACCCCCACCCCTGCCTCGGATGCTTTTCAGCGCAAGCTGGAGGGCTGC
   T P T P A S D A F Q R K L E G C
```

AGGTTCTGTCATGGCTACCATCGCTTCATGCACTCAGTGGGGCGGGTCTT
R F L H G Y H R F M H S V G R V F
CAGCAAGTGGGGGGAGAGCCCGAACCGGAGCCGGAGACACAGCCCCCACC
S K W G E S P N R S R R H S P H Q
5 AGGCCCTGAGGAAGGGGTGCGCAGGACCAGACCTCCAGGAAAGGCAAG
A L R K G V R R T R P S R K G K
AGACTCATGACCAGGGGACAGCTGCCCCGGTAGCCTCGAGAGCACCCCTT
R L M T R G Q L P R
GCCGGTGAAGGATGCGGCAGGTGCTCTGTGGATGAGAGGA
10 *
ACCATCGCAGGATGACAGCTCCCGGGTCCCCAAACCTGTTCCCCTCTGCT
ACTAGCCACTGAGAAGTGCACCTTAAAGAGGTGGGAGCTGGGCAGACCCCT
CTACCTCCTCCAGGCTGGGAGACAGAGTCAGGCTGTTGCGCTCCACCTC
AGCCCCAAGTTCCCCAGGCCAGTGGGGTGGCCGGGCGGGCCACGCGGGA
CCGACTTTCATTGATTGAGGGGTCTGATGACACAGGCTGACTCATGGCC
15 GGGCTGACTGCCCCCTGCCTTGCTCCCCGAGGCCTGCCGGTCCCTCCCT
CTCATTGACTTGCAAGGCCGTTGCCCCAGACTTCCTCCTTTCCGTGTTT
CTGAAGGGGAGGTACAGCCTGAGCTGGCCTCCTATGCCTCATCATGTCC
CAAACCAGACACCTGGATGTCTGGGTGACCTCACTTTAGGCAGCTGTAAC
AGCGGCAGGGTGTCCCAGGAGCCCTGATCCGGGGGTCCAGGGAATGGAGC
TCAGGTCCCAGGCCAGCCCCGAAGTCGCCACGTGGCCTGGGGCAGGTCAC
TTTACCTCTGTGGACCTGTTTTCTCTTTGTGAAGCTAGGGAGTTAGAGGC
20 TGTACAAGGCCCCCACTGCCTGTGCGTTGCTTGGATTCCCTGACGTAAGG
TGGATATTAATAATCTGTAAATCAGGACAGGTGGTGAAATGGCGCTGGG
AGGTGTACACGGAGGTCTCTGTAAAAGCAGACCCACCTCCCAGCGCCGGG
AAGCCCGTCTTGGGTCCCTCGCTGCTGGCTGCTCCCCCTGGTGGTGGATCC
TGGAATTTTCTACGCAGGAGCCATTGCTCTCCTAGAGGGGTCTCAGAA
ACTGCGAGGCCAGTTCCTTGGAGGGACATGACTAATTTATCGATTTTTAT
CAATTTTTATCAGTTTTATATTTATAAGCCTTATTTATGATGTATATTTA
25 ATGTTAATATTGTGCAAACCTATATTTAAACCTTGCCTGGTTTCTAA

The consensus sequence was further verified by
comparison with the sequence of the genomic clone. The
open reading frame continues from nucleotide 1 to the
30 stop codon at nucleotide 783. The open reading frame
codes for 8 amino acids upstream from the putative ini-
tiating methionine. The nucleotide sequence coding for
the putative initiating methionine agrees with the
consensus sequence for the initiating methionine
35 (Kozak, Cell (1986) 44:283-292).

The amino acid sequence of the Oncostatin M polypeptide deduced from the consensus cDNA sequence shows that Oncostatin M is derived from a 253 amino acid precursor polypeptide. The amino terminal
5 sequence of purified Oncostatin M (see above and and Zarling, et al., Proc. Natl. Acad. Sci. USA (1986) 83:9739-9743) occurs at amino acid 26. It is preceded
A by a hydrophobic region that appears to function as a signal sequence. The mature protein has 228 amino
10 acids with a molecular weight of 26,000 which is in close agreement with the approximate $M_r = 28,000$ as determined by the polyacrylamide gel electrophoresis of the purified Oncostatin M (Zarling, et al., 1986 supra).

15 Earlier protein chemistry work (Zarling et al., 1986 supra) showed that Oncostatin M is a glycoprotein. The cDNA clone sequence suggests two potential N-glycosylation sites (Hubbard and Ivatt, Ann. Rev. Biochem. (1981) 50:555-583) located at amino acids
20 76 and 193 of the mature protein. The nucleotide-derived protein sequence shows that Oncostatin M is an extremely hydrophilic molecule. Twenty four base pairs of the 5' untranslated region and 1054 base pairs of 3' untranslated regions were obtained in the different
25 cDNA clones. However, a polyA tail and polyadenylation recognition site were not obtained.

4. Preparation of pOncMVV2

The 2.1 kb Oncostatin M cDNA was excised from the λ phage recombinant, pOncM46, by EcoRI digestion.
30 The insert was cloned into pEMBL18 (Dente et al., Nucl. Acid Res. (1983) 11:1645-1655) at the EcoRI site to produce clone pOncM46-15 vector with the Oncostatin M coding sequence opposed to the β -gal sequence. The 5' noncoding sequence of Oncostatin M was removed by SalI
35 and BglII double digestion of pOncM46-15 and replaced by a synthetic 80 bp SalI-BglII fragment to provide new BamHI and NcoI sites. The resulting clone was termed

pOncMEV5. The sequence of the 80 bp fragment is as follows:

5 5' - TCGACGGATCCACCATGGCGGCGATCGGCAGCTGCTCG
 3' - GCCTAGGTGGTACCGCCGCTAGCCGTCGACGAGC

AAAGAGTACCGCGTGCTCCTTGGCCAGCTCCAGAAGCAGACA - 3'
TTTCTCATGGCGCACGAGGAACCGGTCGAGGTCTTCGTCTGTCTAG - 5

10 The coding sequence of Oncostatin M was
excised from pOncMEV5 by XhoI and SalI double digestion
as a 0.7 kb fragment and cloned into the pUC8 vector as
the SalI site. The clone pOncMVV2, containing the cDNA
15 insert with the coding sequence opposed to the lacZ'
sequence, was isolated. The 0.7 kb NcoI-BamHI and the
0.7 kb BamHI-BamHI fragment excised from clone pOncMVV2
were used to construct a λ PL-based (pBM16/NDP/OncM)
expression vector.

20

Example IV.

Expression of the Polypeptide of Interest as a Fusion Protein with the N-Protein

A. Modified Synthetic TGF

25

1. Preparation of pBM11/N/TGF

The modified human TGF was expressed in this
system as part of a fusion with the 33 N-terminal amino
acids of the N-gene and has the sequence QEEK replacing
the human sequence QEDK.

30

a. Preparation of a 780 bp SphI-PvuI fragment of pBM11/N/TTV

Plasmid pBM11/N/TTV was digested with SphI and
PvuI and the 780 bp SphI-PvuI fragment was gel puri-
fied. This fragment contains part of the pBM11 plasmid

35

at the PvuI end and at the SphI end, the N-gene and N-terminal two-thirds of the human TGF gene.

b. Preparation of the 5 kb BamHI-PvuI fragment of pBM11/N/TTV

5 Plasmid pBM11/N/TTV was digested with BamHI and PvuI and the 5 kb BamHI-PvuI fragment was gel purified.

c. Ligation and isolation of pBM11/N/TGF:

10 Oligonucleotides TGF 205 and 206, the 780 bp SphI-PvuI fragment and the 5 kb BamHI-PvuI fragment of pBM11/N/TTV were ligated together and used to transform competent HB101. The transformants were selected on neomycin and were screened by restriction analysis using EcoRI and nucleotide sequencing following the
15 Sanger-dideoxy method. A correct construction was isolated and denoted pBM11/N/TGF.

N-gene →
20 ATGGATGCACAAACACGCCGCCGCGAACGTCGCGCAGAGAAACAGGCTCAATGGA
M D A Q T R R E R R A E K Q A Q W K

BamHI
AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAAACAGTTCGGATCCGCAT
A A N P L L V G V S A K P V R I R M

25 TGF →
GGTTGTTTCTCACTTTAACGACTGCCCGGACTCTCATACTCAGTTTTGCTTTCAT
V V S H F N D C P D S H T Q F C F H

KpnI SphI
30 GGTACCTGCCGTTTTCTGGTTCAGGAAGAAAAACCGGCATGCGTTTGCCATTCTG
G T C R F L V Q E E K P A C V C H S G

BamHI
GCTAGGTTGGCGCACGTTGCGAACACGCTGACCTGCTGGCTTAAGGATCC
Y V G A R C E H A D L L A Ter

35

B. Modified Synthetic TGF-VGF Hybrid

1. Preparation of pBM11/N/TTV

In this construct, a synthetic modified TTV chimeric gene was expressed as the C-terminal portion of a fusion protein having the first 33 amino acids of the N-gene at the N-terminus. This hybrid growth factor contained the amino acid sequence of human TGF in the amino terminal two-thirds of the gene with the exception of the sequence QEEK which was altered from the natural human sequence QEDK. The carboxy terminus was derived from the amino acid sequence of VGF and terminated with the sequence YQR upstream of the natural sequence PNT.

15 a. Preparation of NcoI(blunt)-BamHI TTV synthetic gene

Plasmid pLEBam/TTV was digested with NcoI and the ends were made blunt by filling in the overhangs using the Klenow fragment of DNA polymerase. The DNA was then digested with BamHI and the 170 bp NcoI(blunt)-BamHI TTV fragment was gel purified.

20 b. Preparation of BamHI digested pBM11

Plasmid pBM11 was digested with BamHI.

c. Ligation and isolation of pBM11/N/TTV

BamHI digested pBM11, the NcoI(blunt)-BamHI TTV fragment, and BamHI linkers (5'GATCCG3') were ligated together using DNA ligase and the resulting mixture was used to transform competent HB101. The transformants were selected on neomycin and were screened using restriction analysis and nucleotide sequencing using the Sanger-dideoxy method. A correct construct was isolated and denoted pBM11/N/TTV.

35 N-gene →
ATGGATGCACAAACACGCCGCCGCGAACGTCGCGCAGAGAAACAGGCTCAATGGA
M D A Q T R R R E R R A E K Q A Q W K

AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAACAGTTCGGATCCGCAT
A A N P L L V G V S A K P V R I R M

5 TGF +
GGTTGTTTCTCACTTTAACGACTGCCCGGACTCTCATACTCAGTTTTGCTTTCAT
V V S H F N D C P D S H T Q F C F H

GGTACCTGCGT TTTCTGGTTCAGGAAGAAAAACCGGCATGCGTTTGCTCTCATG
G T C R F L V Q E E K P A C V C S H G

EcoRI
GCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGGTCGACTACCAGCGTTAAG
Y T G I R C Q H V V L V D Y Q R Ter

15 BamHI
GATCC

C. Synthetic Platelet Factor 4

20 1. Preparation of pBM11/N/PF4 (N-gene/Platelet
Factor 4)

This plasmid was prepared as described above for pBM11/N/TTV, except that the synthetic PF4 gene was used in place of the chimeric TTV gene. The nucleotide sequence and corresponding amino acid sequence of the synthetic platelet factor 4 gene in fusion downstream of the nucleotide sequences coding for the first 33 amino acids of the bacteriophage λ N-gene in the expression vector pBM11 is as follows.

30

N-gene ->
M D A Q T R R R E R R A E K
ATG GAT GCA CAA ACA CGC CGC CGC GAA CGT CGC GCA GAG AAA

35 Q A O W K A A N P L L V G V
CAG GCT CAA TGG AAA GCA GCA AAT CCC CTG TTG GTT GGG GTA

PF4 ->

S A K P V R I R M E A E E D
 AGC GCA AAA CCA GTT CGG ATC CGC ATG GAA GCT GAA GAG GAT

5 G D L Q C L C V K T T S Q V
 GGA GAT CTG CAA TGC CTG TGC GTT AAG ACT ACG TCT CAG GTT

R P R H I T S L E V I K A G
 AGA CCG CGG CAT ATC ACT AGC CTC GAG GTT ATC AAA GCG GGC

10 P H C P T A Q L I A T L K N
 CCA CAC TGT CCG ACT GCG CAG CTG ATC GCG ACT CTG AAA AAC

G R K I C L D L Q A P L Y K
 GGC CGT AAA ATA TGT CTG GAT CTG CAG GCA CCG CTG TAC AAG

15 K I I K K L L E S ***
 AAA ATC ATC AAA AAG CTT CTC GAG TCT TGA

Example V.

20 Preparation of the Polypeptide of Interest
 as a Fusion Protein
with the N-Protein and a Cleavage Site

A. Modified Synthetic VGF

1. Preparation of pBM11/NDP/VGFA:

25 The N-terminal sequence of the synthetic VGFA
 gene is a truncated version of the natural VGF sequence
 and begins with the sequence DIPAIR. In this plasmid
 the VGFA fragment is located downstream of 32 amino
 acids of the lambda N-protein and the dipeptide
 30 aspartic acid-proline. In order to preserve the KpnI
 cloning site, the synthetic sequence was altered to
 code for CLHCGTC instead of the natural VGF sequence
 CLHGDC and terminates with the sequence YQR upstream of
~~the natural sequence PNT.~~ In addition, the VGFA gene
 35 codes for the sequence GYACVC which replaces the
 natural sequence GMYCRC.

a. Preparation of a KpnI-BamHI 80bp C-terminal fragment of the synthetic VGF gene

Plasmid pLEBam/TVVi was digested with KpnI and BamHI and the 80bp KpnI-BamHI fragment was gel
5 purified. This fragment contains the C-terminal two-thirds of the synthetic VGF gene with the KpnI site at the 5' end.

b. Preparation of BamHI digested dephosphorylated pBM11

10 Plasmid pBM11/N/TTV was digested with BamHI and the 5' phosphates were removed by treatment with calf intestinal alkaline phosphatase. The 5.6kp BamHI plasmid fragment was gel purified.

c. Ligation and isolation of pBM11/NDP/VGFA

15 Oligonucleotides VGF 103a, 104a, the 5.6kb BamHI fragment of pBM11 and the 80bp KpnI-BamHI fragment of pLEBam/TVV were ligated together using DNA ligase and then used to transform competent HB101. The transformants were selected on neomycin and were
20 screened by restriction analysis using ClaI and nucleotide sequencing following the Sanger-dideoxy technique. A correct construction was isolated and denoted pBM11/NDP/VGFA. This construction has the sequences GTC and GYACVC instead of the authentic VGF
25 sequences GDC and GMYCRC.

N-gene +

ATGGATGCACAAACACGCCGCCGGAACGTCGCGCAGAGAAACAGGCTCAATGGA
30 M D A Q T R R R E R R A E K Q A Q W K

ClaI

AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAACAGTTCCGGATCGATC
A A N P L L V G V S A K P V R I D P

35

NcoI VGF +

CCATGGACATCCCGGCTATCCGTCTGTGCGGCCCGGAAGGCGACGGCTACTGCCT
M D I P A I R L C G P E G D G Y C L

KpnI
SphI
 GCATGGTACCTGCATCCATGCACGTGACATCGACGGCTACGCATGCGTTTGCTCT
 H G T C I H A R D I D G Y A C V C S

5

EcoRI
 CATGGCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGGTCGACTACCAGCGT
 H G Y T G I R C Q H V V L V D Y Q R

BamHI
 10 TAAGGATCC
 Ter

2. Preparation of pBM11/NDP/VGFA

15 The N-terminal sequence of VGFA is a truncated
 version of the natural VGF sequence and starts with the
 sequence DIPAIR. In addition, the VGFA sequence con-
 tains the altered sequences GTC and GYACRC instead of
 the natural VGF sequences GDC and GMYCRC. In this
 20 plasmid the VGFA gene is located downstream of 32 amino
 acids of the lambda N-protein and the dipeptide
 aspartic acid-proline. Treatment of the purified
 fusion protein with formic acid results in cleavage at
 the acid labile aspartic acid-proline peptide bond
 allowing separation of the VGFA protein from the lambda
 25 N-protein amino-terminus. Cleavage is such that the
 VGFA protein is left with the proline residue at the
 amino terminus.

a. Preparation of SphI digested, dephosphorylated pBM11/DP/VGFA

30 Plasmid pBM11/DP/VGFA (10 µg) was digested
 with 30 units of SphI and the 5' phosphates were
 removed by treatment with calf intestinal alkaline
 phosphatase. The 5kb plasmid fragment was recovered
 after electrophoresis on an agarose gel.

35

b. Preparation of an EcoRI-SphI 70bp
fragment of pBM11/DP/VGFA

Plasmid pBM11/DP/VGFA (10 µg) was digested
with 30 units of EcoRI and then 30 units of SphI. The
5 70bp fragment was recovered after electrophoresis on an
agarose gel.

3. Ligation and isolation of pBM11/NDP/VGFA

The 24bp fragment containing oligonucleotides
VGf 1A and 2A, the 5kb SphI fragment and the 70bp
10 EcoRI-SphI fragment of pBM11/DP/VGFA were ligated
together and the mixture was used to transform
competent E. coli HB101 cells. The transformants were
screened by nucleotide sequencing using the Sanger-
dideoxy nucleotide method. A correct clone was isolated
15 and denoted pBM11/NDP/VGFA.

N-gene +

ATGGATGCACAAACACGCCGCCGCGAACGTCGCGCAGAGAAACAGGCTCAATGGA
M D A Q T R R R E R R A E K Q A Q W K

20

ClaI

AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAAACCAGTTCGGATCGATCC
A A N P L L V G V S A K P V R I D P

25

NcoI VGf +

CCATGGACATCCCGGCTATCCGTCTGTGCGGCCCGGAAGGCGACGGCTACTGCCT
M D I P A I R L C G P E G D G Y C L

KpnI

SphI

GCATGGTACCTGCATCCATGCACGTGACATCGACGGCTACGCATGCCGTTGCTCT
30 H G T C I H A R D I D G Y A C R C S

EcoRI

CATGGCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGGTCGACTACCAGCGT
H G Y T G I R C Q H V V L V D Y Q R

35

BamHI

TAAGGATCC

Ter

B. Modified Synthetic TGF-VGF Hybrids

1. Preparation of pBM11/NDP/TTV

5 In this construct, the synthetic modified TTV
chimeric gene is expressed as the C-terminal portion of
a fusion protein having the first 32 amino acids of the
N-gene at the N-terminus. An acid labile aspartic
acid-proline dipeptide separates the two parts of the
10 fusion. The hybrid growth factor contains the amino
acid sequence of human TGF in the amino terminal two-
thirds of the gene with the exception of the sequence
QEEK which was altered from the natural human TMR se-
quence QEDK. The carboxy terminus was derived from the
15 amino acid sequence of VGF and terminated with the se-
quence YQR upstream of the natural sequence PNT.

a. Preparation of 5kb NcoI pBM11 plasmid fragment

Plasmid pBM11/NDP/VGFA was digested with NcoI
20 and the 5kb NcoI plasmid fragment was gel purified.
This fragment has one NcoI overhang at the aspartic
acid-proline cleavage site downstream of the sequences
coding for the first 32 amino acids of the N-gene. The
other NcoI site is in the neomycin resistance gene.

25 b. Preparation of 0.6kb NcoI-BamHI pBM11 plasmid fragment

Plasmid pBM11/N/TTV was digested with NcoI and
BamHI and the 0.6kb NcoI-BamHI plasmid fragment was gel
purified. This fragment has the NcoI overhang in the
30 neomycin resistance gene.

c. Preparation of the 170bp synthetic TGF/TGF/VGF fragment

Plasmid pLEBam/TTV was digested with NcoI and
BamHI and the NcoI-BamHI 170bp fragment containing the
35 TGF/TGF/VGF synthetic gene was gel purified. This
fragment has the NcoI overhang at the 5' end of the
gene and the BamHI overhang at the 3' end of the gene.

d. Ligation and isolation of pBM11/NDP/TTV

The 5kb NcoI and the 0.6kb NcoI-BamHI plasmid fragments were ligated with the 170bp NcoI-BamHI TTV gene using DNA ligase and the resulting mixture was used to transform competent HB101. The transformants were selected on neomycin such that only colonies with correctly reconstructed neomycin resistance genes would survive. Transformants were screened using restriction analysis with NcoI and nucleotide sequencing using the Sanger-dideoxy technique. A correct construction was isolated and denoted pBM11/NDP/TTV.

N-gene +
 15 ATGGATGCACAAACACGCCGCCGCGAACGTCGCGCAGAGAAACAGGCTCAATGGA
 M D A Q T R R R E R R A E K Q A Q W K

ClaI
 AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAACAGTTCGGATCGATC
 A A N P L L V G V S A K P -V R I D P

20 NcoI TGF +
 CCATGGTTGTTTCTCACTTTAACGACTGCCCCGACTCTCATACTCAGTTTTGCTT
 M V V S H F N D C P D S H T Q F C F

KpnI SphI VGF +
 25 TCATGGTACCTGCCGTTTTCTGGTTCAGGAAGAAAAACCGGCATGCGTTTGCTCT
 H G T C R F L V Q E E K P A C V C S

EcoRI
 CATGGCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGGTCGACTACCAGCGT
 H G Y T G I R C Q H V V L V D Y Q R

30 BamHI
 TAAGGATCC
 Ter

35 2. Preparation of pBM11/NDP/VTV

In this construct, the synthetic modified VTV chimeric gene was expressed as the C-terminal portion

of a fusion protein having the first 32 amino acids of the N-gene at the N-terminus. An acid labile aspartic acid-proline dipeptide separates the two parts of the fusion. The hybrid growth factor contained the amino acid sequence of human TGF in the middle domain with the amino acid sequence QEEK replacing the natural sequence QEDK. The N-terminal and C-terminal domains were derived from the truncated VGF sequence and begin with the sequence DIPAIR and end with the sequence YQR which is upstream of the natural sequence PNT.

a. Preparation of a 5kb BamHI-NcoI fragment of pBM11

Plasmid pBM11/N/TTV was digested with BamHI and NcoI and the 5kb BamHI-NcoI fragment was gel purified. This fragment contains a BamHI overhang at the 3' end of the sequences coding for the first 32 amino acids of the N-gene and a NcoI site in the neomycin resistance gene.

b. Preparation of a 700 bp KpnI-NcoI fragment of pBM11/N/TTV

Plasmid pBM11/N/TTV was digested with KpnI and NcoI and the 700 bp KpnI-NcoI fragment was gel purified. This fragment is made up of part of plasmid pBM11 containing part of the neomycin resistance gene at the NcoI overhang, and the C-terminal VGF domain of the TTV synthetic gene at the KpnI overhang.

c. Ligation and isolation of pBM11/NDP/VTV

Oligonucleotides VGF 103a and 104a, the 5kb BamHI-NcoI fragment of pBM11 and the 700 bp KpnI-NcoI fragment of pBM11/N/TTV were ligated together using DNA ligase and then used to transform competent HB101. The transformants were selected on neomycin and were screened by restriction analysis using ClaI and nucleotide sequencing following the Sanger-dideoxy technique.

N-gene +
 ATGGATGCACAAACACGCCGCCGCGAACGTCGCGCAGAGAAACAGGCTCAATGGA
 M D A Q T R R R E R R A E K Q A Q W K

5 AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAAACAGTTCGGATCGATC
 A A N P L L V G V S A K P V R I D P

10 NcoI VGF +
 CCATGGACATCCCGGCTATCCGTCTGTGCGGCCCGGAAGGCGACGGCTACTGCCT
 M D I P A I R L C G P E G D G Y C L

KpnI TGF + SphI VGF +
 GCATGGTACCTGCCGTTTCTGGTTCAGGAAGAAAAACCGGCATGCGTTTGCTCT
 H G T C R F L V Q E E K P A C V C S

15 EcoRI
 CATGGCTACACTGGAATTCGTTTCGCAGCATGTTGTTCTGGTTCGACTACCAGCGT
 H G Y T G I R C Q H V V L V D Y Q R

20 BamHI
 TAAGGATCC
 Ter

3. Preparation of pBM16/NDP/TVV

25 In this construct, the synthetic modified TVV
 chimeric gene was expressed as the C-terminal portion
 of a fusion protein having the first 32 amino acids of
 the N-gene at the N-terminus. An acid labile aspartic
 acid-proline dipeptide separates the two parts of the
 fusion. The hybrid growth factor contained the amino
 30 acid sequence of human TGF in the N-terminal domain.
 The middle and C-terminal domains were derived from the
 truncated VGF sequence and end with the sequence YQR.
 In addition, the synthetic gene has the modification
 GYACVC for GMYCRC.

35

a. Preparation of 4.3kb NcoI-BglII fragment
of pBM11/NDP/VGFa

Plasmid pBM11/NDP/VGFa was digested with NcoI
and BglII and the 4.3kb fragment was gel purified. The
5 NcoI overhang is positioned at the aspartic acid-
proline cleavage site just downstream of the first 32
amino acids of the N-gene.

b. Preparation of the 1.2kb BamHI-BglII
fragment of pBM11M5

10 Plasmid pBM11M5 was digested with BamHI and
BglII and the 1.2kb fragment was gel purified. This
fragment differs from the normal pBM11 fragment in that
the NcoI site in the neomycin resistance gene has been
removed, and all subsequent vectors lacking this NcoI
15 site are referred to as pBM16.

c. Preparation of the 170bp NcoI-BamHI TVV
synthetic gene

Plasmid pLEBam/TVV was digested with NcoI and
BamHI and the 170 bp NcoI-BamHI fragment was gel
20 purified. This synthetic gene fragment has the NcoI
site at the 5'-end and the BamHI site at the 3'-end.

d. Ligation and isolation of pBM16/NDP/TVV

The 4.3kb NcoI-BglII fragment of
pBM11/NDP/VGFa and 1.2kb BamHI-BglII fragment of
25 pBM11M5, and the 170 bp NcoI-BamHI TVV synthetic gene
fragment were ligated together using DNA ligase and the
resulting mixture was used to transform competent
HB101. The transformants were selected on neomycin and
screened by restriction analysis and nucleotide
30 sequencing using the Sanger-dideoxy technique. The
plasmid is denoted pBM16 to indicate the loss of the
NcoI restriction site in the neomycin resistance gene.

35 N-gene +
ATGGATGCACAAACACGCCGCCGCGAACGTCGCGCAGAGAAACAGGCTCAATGGA
M D A Q T R R R E R R A E K Q A Q W K

ClaI

AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAACCAGTTCGGATCGATC
 A A N P L L V G V S A K P V R I D P

5

NcoI TGF +

CCATGGTTGTTTCTCACTTTAACGACTGCCCGGACTCTCATACTCAGTTTTGCTT
 M V V S H F N D C P D S H T Q F C F

?

8

KpnI VGF +

SphI

10

TCATGGTACCTGCATCCATGCACGTGACATCGACGGCTACGCATGCGTTTGCTCT
 H G T C I H A R D I D G Y A C V C S

EcoRI

CATGGCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGGTCGACTACCAGCGT
 H G Y T G I R C Q H V V L V D Y Q R

15

BamHI

TAAGGATCC

Ter

20 C. Synthetic EGF1. Preparation of pBM11/NDP/EGF

In this construct the human EGF gene is expressed as part of a fusion with the 32 N-terminal amino acids of the N-gene which is downstream of an Asp-Pro cleavage site.

25

a. Preparation of a 5kb NcoI fragment of pBM11

Plasmid pBM11/DP/VGFA was digested with NcoI and the 5' phosphates were removed by treatment with calf alkaline intestinal phosphatase. The 5kb plasmid fragment was gel purified. This fragment has one NcoI overhang at the Asp-Pro cleavage site downstream of the sequences coding for the first 32 amino acids of the N-gene. The other NcoI site is in the Neomycin resistance gene.

35

b. Preparation of a 0.6kb NcoI-BamHI
fragment of pBM11

Plasmid pBM11/N/TTV was digested with NcoI and
BamHI and the 0.6kb NcoI-BamHI plasmid fragment was gel
5 purified. This fragment has the NcoI overhang in the
Neomycin resistance gene.

c. Ligation and Isolation of pBM11/DP/EGF

The three sets of annealed EGF
oligonucleotides with an NcoI overhang at the 5' end
10 and a BamHI overhang at the 3' end, the 5kb NcoI
fragment of pBM11 and the 0.6kb NcoI-BamHI fragment of
pBM11 were ligated together using T4 DNA Ligase and the
resulting mixture was used to transform competent E.
coli HB101. The transformants were selected on
15 Neomycin such that only colonies with a correctly
reconstructed Neomycin resistance gene would survive.
The transformants were screened by restriction analysis
using EcoRI and BamHI and by DNA sequencing, as
described above.

20

N-gene +

ATGGATGCACAAACACGCCGCCGCGAACGTCGCGCAGAGAAACAGCGTCAATGGA
M D A Q T R R R E R R A E K Q A Q W K

25

AAGCAGCAAATCCCCTGTTGGTTGGGGTAAGCGCAAAACAGTTCGGATCGATCC
A A N P L L V G V S A K P V R I D P

EGF1 + EcoRI

CATGAATTCTGACTCTGAATGCCCCGCTGTCTCATGACGGCTACTGCCTGCATGAC
30 M N S D S E C P L S H D G Y C L H D

EGF2 +

EGF3 +

NsiISphI

GGCGTATGCATGTACATCGAAGCTCTGGACAAGTACGCATGCAACTGCGTTGTTG
G V C M Y I E A L D K Y A C N C V V G

35

GCTACATCGGCCGAACGTTGCCAGTACCGTGACCTGAAATGGTGGGAAGTGCCTTA
Y I G E R C Q Y R D L K W W. E L R *

5

D. Synthetic Platelet Factor 41. Preparation of pBM11/NDP/PF4 (N-gene/DP/
Platelet Factor 4)

10 In this construct, the synthetic PF4 gene is expressed as the C-terminal portion of a fusion protein having the first 32 amino acids of the N-gene at the N-terminus. An acid labile aspartic acid-proline dipeptide separates the two parts of the fusion.

a. Preparation of 5kb NcoI pBM11 plasmid
15 fragment

Plasmid pBM11/NDP/VGFA was digested with NcoI and the 5kb NcoI plasmid fragment was gel purified. This fragment has one NcoI overhang at the aspartic acid-proline cleavage site downstream of the sequences
20 coding for the first 32 amino acids of the N-gene. The other NcoI site is in the neomycin resistance gene.

b. Preparation of 0.6kb NcoI-BamHI pBM11
plasmid fragment

Plasmid pBM11/N/PF4 was digested with NcoI and
25 BamHI and the 0.6kb NcoI-BamHI plasmid fragment was gel purified. This fragment has the NcoI overhang in the neomycin resistance gene.

c. Ligation and isolation of pBM11/NDP/PF4

30 The 5kb NcoI and the 0.6kb NcoI-BamHI plasmid fragments were ligated with the PF4 gene using DNA ligase and the resulting mixture was used to transform competent HB101. The transformants were selected on neomycin such that only colonies with correctly re-constructed neomycin resistance genes would survive.
35 Transformants were screened using restriction analysis with NcoI and nucleotide sequencing using the Sanger-

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dideoxy technique. A correct construction was isolated and denoted pBM11/NDP/PF4.

The nucleotide sequence and corresponding amino acid sequence of the synthetic platelet factor 4 gene in fusion downstream of the nucleotide sequences coding for the first 32 amino acids of the bacteriophage λ N-gene and the acid labile dipeptide Asp-Pro (***) in the expression vector pBM11 is as follows.

10

N-gene ->

M D A Q T R R R E R R A E K
ATG GAT GCA CAA ACA CGC CGC CGC GAA CGT CGC GCA GAG AAA

15

Q A Q W K A A N P L L V G V
CAG GCT CAA TGG AAA GCA GCA AAT CCC CTG TTG GTT GGG GTA

20

*** ** PF4 ->
S A K P V R I D P M E A E E
AGC GCA AAA CCA GTT CGG ATC GAT CCC ATG GAA GCT GAA GAG

D G D L Q C L C V K T T S Q
GAT GGA GAT CTG CAA TGC CTG TGC GTT AAG ACT ACG TCT CAG

V R P R H I T S L E V I K A
GTT AGA CCG CGG CAT ATC ACT AGC CTC GAG GTT ATC AAA GCG

G P H C P T A Q L I A T L K
GGC CCA CAC TGT CCG ACT GCG CAG CTG ATC GCG ACT CTG AAA

25

N G R K I C L D L Q A P L Y
AAC GGC CGT AAA ATA TGT CTG GAT CTG CAG GCA CCG CTG TAC

K K I I K K L L E S ***
AAG AAA ATC ATC AAA AAG CTT CTC GAG TCT TGA

30

E. Oncostatin M

1. Construction of pBM16/NDP/OncM

a. Preparation of modified Oncostatin M gene fragment

Plasmid pOncMVV2 containing a modified Oncostatin M gene was digested with NcoI and BamHI and the 700 bp NcoI-BamHI Oncostatin M gene was gel purified. This fragment contained the NcoI overhang at the 5' end

of the gene and the BamHI overhang at the 3' end of the gene.

b. Ligation and isolation of pBM16/NDP/OncM

The 700 bp NcoI-BamHI fragment of the modified Oncostatin M gene and the NcoI-BamHI fragment of the plasmid pBM16/NDP were ligated together with T4 ligase and transformed into competent HB101 E. coli. The transformants were screened for correct construction by nucleotide sequencing using the Sanger-dideoxy technique. A correct colony was chosen and termed pBM16/NDP/OncM.

c. Preparation of Oncostatin M using pBM16/NDP/OncM

E. coli HB101 strain harboring the plasmid pBM16/NDP/OncM, which encodes the first 32 amino acids of the bacteriophage λ N-gene fused to an acid cleavable dipeptide (DP), and the synthetic OncM gene was grown at 30°C. At an OD₆₀₀ of approximately 0.9 the temperature was raised to 42°C which inactivates the temperature sensitive repressor inducing the PL promoter to allow transcription and translation of the NDP/OncM fusion gene. The fusion protein is characterized by having the 32 amino-terminal residues of the bacteriophage λ N-gene followed by the acid labile dipeptide Asp-Pro followed by the 228 amino acids of Oncostatin M including its N-terminal methionine.

2. Preparation of Oncostatin M Using pBMX

An 82 bp fragment was synthesized chemically as shown below.

5' - CATGGCCATTGAAGGGCGCGCTGCGATCGGCAGCTGCTCGAAA
3' - CGGTAACTTCCCGCGGACGCTAGCCGTCGACGAGCTTT

GAGTACCGCGTGCTCCTTGGCCAGCTCCAGAAGCAGACA - 3'
CTCATGGCGCACGAGGAACCGGTTCGAGGTCTTCGTCTGTCTAG - 5'

The 82 bp fragment and truncated OncM cDNA (BglIII-HindIII) isolated from pOncMVV2 were cloned into the pBM16/NDP/TVV vector prepared by NcoI and HindIII double digestion. The DNA was used to transform E. coli DH5 α . The clone pBMX was isolated and confirmed to carry the predicted sequence. Since the coding sequence contains the -I-E-G-R-, a factor X recognition site, precisely fused to the N-terminal of mature OncM, the recombinant protein produced by pBMX should be cleaved at the R residue of -I-E-G-R- to generate mature OncM with the authentic N-terminal sequence following treatment of activated factor X. Similar procedures were used to prepare Oncostatin M using pBMX as to prepare Oncostatin M from pBM16/NDP/OncM.

Example VI.

Preparation of the Polypeptide of Interest as a Fusion Protein with the Alkaline Phosphatase Signal Sequence

A. Preparation of pBM11/PAD/EGF

Synthetic oligonucleotides were designed to allow insertion of DNA coding for a modified alkaline phosphatase signal peptide and a linker region with 3 cloning sites (HindIII, SmaI and BamHI) into the pBM11 expression vector downstream of the P_L promoter and N gene ribosomal binding site. The nucleotide sequence was optimized to be as similar as possible to the nucleotide sequence of the amino terminus of the lambda N gene as the lambda N gene sequence has evolved with that of its ribosomal binding site for efficient ribosome initiation and translation. In addition, the second amino acid of the alkaline phosphatase signal sequence, the basic amino acid lysine was changed to an acidic amino acid, aspartic acid.

1. Preparation of 0.17kb EcoR I-BamHI fragment of EGF

Plasmid pBM11/NDP/EGF (30ug) was digested with 30 units of EcoRI and then treated with 4 units of Klenow fragment of DNA polymerase to create blunt ends. The DNA was finally digested with 30 units of BamHI and the 0.17kb fragment of the EGF gene was recovered after electrophoresis on an agarose gel. The DNA so purified has a blunted EcoRI site at the 5' end and a BamHI overhang at the 3' end.

2. Preparation of 0.5kb Pvu I-Hind III fragment of pBM11/PAD

Plasmid pBM11/PAD (18ug) was digested with 30 units of HindIII and then treated with Klenow fragment to blunt the ends. The DNA was then digested with PvuI and the 0.5kb PvuI-HindIII (blunt) fragment was recovered after electrophoresis on an agarose gel.

3. Preparation of the 5.2kb Pvu I-BamHI fragment of pBM11/PAD

Plasmid pBM11/PAD (18ug) was digested with 30 units of PvuI followed by 30 units of BamHI. The 5.2kb fragment was recovered after electrophoresis on an agarose gel.

4. Ligation and isolation of pBM11/PAD/EGF

The 0.17 kb EcoRI (blunt)-BamHI fragment, the 0.5kb PvuI-HindIII (blunt) fragment, and the 5.2kb PvuI-BamHI fragment were ligated together and the resulting mixture was used to transform competent E. coli HB101. The transformants were screened using DNA sequencing, as described above. The desired signal sequence/EGF region had the following sequence:

Signal Sequence
ATGGATCAATCTACAATCGCCCTCGCACTTCTCCCACTGCTGTTCCT
M D Q S T I A L A L L P L L F T

EGF

CCAGTGACAAAAGCTAATTCTGACTCTGAATGCCCGCTGTCTCATGAC
P V T K A N S D S E C P L S H D

NsiI

5 GGCTACTGCCTGCATGACGGCGTATGCATGTACATCGAAGCTCTG
G Y C L H D G V C M Y I E A L

SphI

GACAAGTACGCATGCAACTGCGTTGTTGGCTACATCGGCGAACGT
D K Y A C N C V V G Y I G E R

10

BamHI

TGCCAGTACCGTGACCTGAAATGGTGGGAACTGCGTTAAGGATCC
C Q Y R D L K W W E L R *

15 The efficacy of the production of foreign
protein in the pBM11/PAD expression system and the
ability to purify functionally active foreign proteins
from the fusion product has been show using
pBM11/PAD/EGF as an example. After size exclusion
20 chromatography (TSK-250), 10.3 mg of equivalents of
active EGF fusion polypeptide was recovered from 23 g
(8 liters) of E. coli derepressed to express the EGF
gene. Forty percent of the EGF activity was derived
from EDF cleaved from the signal sequence.

25

B. Construction of pBM11/PAD/OncM

1. Preparation of modified Oncostatin M gene fragment

30 Plasmid pOncMVV2 containing a modified Onco-
statin M gene was digested with NcoI and the 5' over-
hanging bases were removed by treatment with S1 nucle-
ase leaving the fragment blunt-ended. The nuclease
treatment removed the codons for the initiating methio-
nine. The plasmid was further digested with BamHI and
35 the 700 bp NcoI(blunt)-BamHI Oncostatin M gene fragment
was gel purified. This fragment contained the NcoI

blunt end at the 5' end of the gene and BamHI overhang at the 3' end of the gene.

2. Preparation of pBM11M3/PAD fragments

Plasmid pBM11M3/PAD containing the nucleotide sequences coding for a modified alkaline phosphatase signal sequence was digested with HindIII which cuts directly downstream of the signal sequence. The overhanging ends were filled in and made blunt using Klenow fragment of DNA polymerase. The resulting DNA was further digested with PvuI and the 680 bp HindIII(blunt)-PvuI fragment was gel purified.

Plasmid pBM11M3/PAD was also digested with BamHI and PvuI and the 5 kb BamHI-PvuI fragment was gel purified.

3. Ligation and isolation of pBM11/PAD/OncM

The 700 bp NcoI(blunt)-BamHI Oncostatin M gene fragment, the 680 bp HindIII(blunt)-PvuI fragment of pBM11M3/PAD and the 5 kb BamHI-PvuI fragment of pBM11M3/PAD were ligated together using T4 ligase and transformed into competent HB101 E. coli. Correct construction was assayed by nucleotide sequencing using the Sanger-dideoxy technique. A correct colony was chosen and designated pBM11/PAD/OncM.

C. Preparation of pBM11/PAD/nVGf

Synthetic oligonucleotides were designed to link the VGf synthetic gene with an alkaline phosphatase modified signal sequence to provide for an optimal signal sequence cleavage site by coding for the additional N-terminal residues occurring immediately downstream of the signal sequence cleavage site in the natural VGf, denoted extreme N-terminus above. The nVGf sequence contains the altered sequences GTC and GYACRC instead of the natural VGf sequences GDC and GMYCRC and terminates with the sequence YQR upstream of the natural sequence PNT. In this expression system, for the majority of the molecules, the signal sequence

remains attached to the nVGfA forming a fusion protein with nVGfA at the C-terminus.

1. Preparation of 0.5kb HindIII-PvuI digested pBM11/PAD

5 Plasmid pBM11/PAD was digested with HindIII and PvuI and the 0.5kb fragment was gel purified. The HindIII site is located at the C-terminus of the modified alkaline phosphatase signal sequence.

10 2. Preparation of the 5.2kb PvuI-BamHI pBM11 plasmid fragment

Plasmid pBM11/NDP/VGfA was digested with PvuI and BamHI and the 5.2kb plasmid fragment was gel purified.

15 3. Preparation of the 170bp NcoI(blunt)-BamHI synthetic VGfA gene

Plasmid pBM11/NDP/VGfA was digested with NcoI and the 5' overhangs were removed by treatment with S1-nuclease. This created a blunt end at the first codon of the VGfA truncated synthetic gene. The DNA was then
20 digested with BamHI and the 170bp NcoI(blunt)-BamHI fragment was gel purified.

4. Ligation and isolation of pBM11/PAD/nVGfA

Oligonucleotides VGf105 and 106, the 0.5kb HindIII-PvuI fragment of pBM11/PAD, the 5.2kb PvuI-BamHI pBM11 fragment and the 170bp NcoI(blunt)-BamHI
25 synthetic VGfA gene were ligated together using DNA ligase and the resulting mixture was used to transform competent HB101. The transformants were selected on neomycin and screened by restriction analysis and
30 nucleotide sequencing using the Sanger-dideoxy technique. A correct construct was isolated containing the modified alkaline phosphatase signal sequence in frame with the nVGfA gene.

Signal Sequence +

ATGGATCAATCTACAATCGCCCTCGCACTTCTCCCACTGCTGTTCACTCCAGTGA
M D Q S T I A L A L L P L L F T P V T

nVGF +

5 CAAAAGCTGACTCTGGTAACGCTATCGAACTACTTCTCCGGAATCACTAACGC
K A D S G N A I E T T S P E I T N A

TACTACTGACATCCCGGCTATCCGTCTGTGCGGCCCGGAAGGCGACGGCTACTGC
T T D I P A I R L C G P E G D G Y C

10

KpnI

SphI

CTGCATGGTACCTGCATCCATGCACGTGACATCGACGGCTACGCATGCCGTTGCT
L H G T C I H A R D I D G Y A C R C S

EcoRI

15 CTCATGGCTACACTGGAATTCGTTGCCAGCATGTTGTTCTGGTCGACTACCAGCG
H G Y T G I R C Q H V V L V D Y Q R

BamHI

TTAAGGATCC

Ter

20

D. Preparation of pBM11/PAD/PF4 (Signal sequence of
alkaline phosphatase with Asp as residue 2 instead
of Lys/Platelet Factor 4).

25 The nucleotide sequence and corresponding
amino acid sequence of the synthetic platelet factor 4
gene in fusion downstream of the nucleotide sequences
coding for a modified alkaline phosphatase signal
peptide were prepared essentially as described above
for pBM11/PAD/nVGf, except that the synthetic PF4 gene
30 was used instead of the synthetic VGf gene in Step
3. The construct isolated is as follows. Predicted
cleavage site is noted with (***).

35

Signal sequence ->

M D Q S T I A L A L L P L L
ATG GAT CAA TCT ACA ATC GCC CTC GCA CTT CTC CCA CTG CTG

5

*** PF4 ->

F T P V T K A E A E E D G D
TTC ACT CCA GTG ACA AAA GCT GAA GCT GAA GAG GAT GGA GAT

L Q C L C V K T T S Q V R P
CTG CAA TGC CTG TGC GTT AAG ACT ACG TCT CAG GTT AGA CCG

10

R H I T S L E V I K A G P H
CGG CAT ATC ACT AGC CTC GAG GTT ATC AAA GCG GGC CCA CAC

C P T A Q L I A T L K N G R
TGT CCG ACT GCG CAG CTG ATC GCG ACT CTG AAA AAC GGC CGT

K I C L D L Q A P L Y K K I
AAA ATA TGT CTG GAT CTG CAG GCA CCG CTG TAC AAG AAA ATC

15

I K K L L E S ***
ATC AAA AAG CTT CTC GAG TCT TGA

Example VII.

20

Expression of the Polypeptide of Interest
as a Fusion Protein
with the Alkaline Phosphatase Signal Sequence

25

- A. Preparation of pBM11/PAK/nVGfA (Alkaline
phosphatase signal sequence/nVGfA with natural VGf
N-terminus and sequences GTC and GYACRC)

30

Plasmid pBM11/PAD/nVGf was mutagenized in
vitro (Morinaga et al., Biotechnology (1984) 2:636-643)
to alter the codons coding for the second amino acid in
the signal sequence, namely to change the Asp (D) codon
to that for Lys (K) the residue found in the natural
sequence. This mutagenesis also introduced a PvuI site
into the signal sequence.

35

Signal Sequence +

ATGGATCAATCTACAATCGCCCTCGCACTTCTCCCACTGCTGTTCACTCCAGTGACAAAA
 M D Q S T I A L A L L P L L F T P V T K

nVGF +

5 GCTGACTCTGGTAACGCTATCGAACTACTTCTCCGGAATCACTAACGCTACTACT
 A D S G N A I E T T S P E I T N A T T

GACATCCCGGCTATCCGTCTGTGCGGCCCGGAAGGCGACGGCTACTGCCTGCATGGT ^{KpnI}
 D I P A I R L C G P E G D G Y C L H G

10

ACCTGCATCCATGCACGTGACATCGACGGCTACGCATGCCGTTGCTCTCATGGCTACACT ^{SphI}
 T C I H A R D I D G Y A C R C S H G Y T

15 ^{EcoRI} GGAATTCGTTGCCAGCATGTTGTTCTGGTCTGACTACCAGCGTTAAGGATCC ^{BamHI}
 G I R C Q H V V L V D Y Q R Ter

B. Preparation of pBM11/PAK/EGF

20 In this expression cassette the EGF gene is part of a fusion with the alkaline phosphatase signal sequence.

Plasmid pBM11/PAD/EGF was mutagenized in vitro to alter the codons coding for the second amino acid in the signal sequence, to change the Asp (D) codon to that for Lys (K) the residue found in the natural sequence. This mutagenesis also introduced a PvuI site into the signal sequence.

30

^{PvuI}
 Signal Sequence +
 ATGAAACAATCTACGATCGCCCTCGCACTTCTCCCACTGCTGTTCACTCCAGTGA
 M K Q S T I A L A L L P L L F T P V T

35 EGF +
 CAAAAGCTAATTCTGACTCTGAATGCCCGCTGTCTCATGACGGCTACTGCCTGCA
 K A N S D S E C P L S H D G Y C L H

NsiI
SphI

TGACGGCGTATGCATGTACATCGAAGCTCTGGACAAGTACGCATGCAACTGCGTT
 D G V C M Y I E A L D K Y A C N C V

5 GTTGGCTACATCGGCGAACGTTGCCAGTACCGTGACCTGAAATGGTGGGAACTGC
 V G Y I G E R C Q Y R D L K W W E L R

BamHI

GTTAAGGATCC

*

10

C. Preparation of TacPak/EGF (alkaline phosphatase
 signal sequence/human EGF)

1. Preparation of Plasmid Fragments

15 Plasmid p135-1 was derived from plasmid pDR540
 (Pharmacia) and contained the Cro gene SD and a BglII
 site downstream of the lac SD. pDR540 is an expression
 vector containing the trp-lac hybrid promoter. p135-1
 was digested with BglII and BamHI and treated with
 20 bacterial alkaline phosphatase.

Plasmid pBM11/PAK/EGF was digested with PvuII
 and BamHI and the ~230 bp fragment coding for part of
 the alkaline phosphatase signal sequence and human EGF
 was isolated.

25 2. Preparation of TacPak1 and TacPak2
 Oligonucleotides

Synthetic oligonucleotides TacPak1 and TacPak2
 were designed with an overhang, compatible with the
BglII site of p135-1 and a PvuII overhang, compatible
 30 with the PvuII site in the alkaline phosphatase/EGF
PvuII/BamHI fragment. The oligonucleotides were
 synthesized on an Applied Biosystems Oligonucleotide
 Synthesizer.

35

```

          BglIII              PvuI
          |                   |
TacPak1  5' GATCTATGAAACAATCTACGAT 3'
TacPak2  3'      AACTTTGTTAGATGC  5'

```

5

3. Ligation and Isolation of TacPak/EGF Clone

The BglIII-BamHI digested p135-1, the 230 bp PAK/EGF fragment and oligonucleotides TacPak1 and TacPak2 were ligated using DNA ligase, transformed into competent HB101 and a correct construct was isolated by DNA sequencing.

(HindIII site of pDR540)

```

      |
15  AAGCTTACTCCC

```

```

      trp-35      (16bp)      lac-10
CATCCCCCTG [TTGACA] ATTAATCATCGGCTCG (TATAATG)

```

20 mRNA 5' lacI binding site lacSD
TGTGG/AATTGTG AGCGGATAACAATTCACAC {AGGA} AACAGGATCACTA

```

      PvuI
croSD (11bp) BglIII
{AGGA} GGTTCAGATCT

```

25

Signal Sequence ->
ATGAAACAATCTACGATCGCCCTCGCACTTCTCCCACTGCTGTTCACTCCAGTGA
M K Q S T I A L A L L P L L F T P V T

EGF ->
30 CAAAAGCTAATTCTGACTCTGAATGCCGCTGTCTCATGACGGCTACTGCCTGCA
K A N S D S E C P L S H D G Y C L H

```

      NsiI              SphI
TGACGGCGTATGCATGTACATCGAAGCTCTGGACAAGTACGCATGCAACTGCGTT
D G V C M Y I E A L D K Y A C N C V

```

35

GTTGGCTACATCGGCGAACGTTGCCAGTACCGTGACCTGAAATGGTGGGAAGTGC
V G Y I G E R C Q Y R D L K W W E L R

BamHI
GTTAAGGATCC

*

5

Example VIII.

Expression of a Polypeptide of Interest as a Fusion
Protein with the Alkaline Phosphatase Signal Sequence
Using an Expression Cassette
Comprising a Transcriptional Termination Region

10

A. Preparation of pTCPt/EGF ([trp-35]16bp[lac-
10][lacSD]11bp[ATG]/alkaline phosphatase
signal/human EGF/trans. term.-NEO)

15

This plasmid is designed to have the tac
promoter elements and utilize the cro SD to express
human EGF behind the alkaline phosphatase signal
sequence. It has a pBR322 background with the Neomycin
resistance gene.

20

1. Preparation of the 420bp HindIII(blunt)-BamHI
fragment of TacPak/EGF

25

TacPak/EGF was digested with HindIII and then
treated with the Klenow fragment of DNA polymerase to
create blunt ends. The DNA was then digested with
BamHI and the 420bp fragment containing the tac
promoter elements and the coding region for the
alkaline phosphatase signal sequence and human EGF was
isolated by agarose gel electrophoresis.

30

2. Preparation of the 2.8kb EcoRI(blunt)-BamHI
fragment of pBM16t/NDP/VGFa

35

pBM16t/NDP/VGFa was digested with EcoRI and
then treated with Klenow to create blunt ends. The DNA
was then digested with BamHI and the 2.8 kb fragment
was isolated. This DNA fragment contains the pBR322
origin, the neomycin resistance gene with its NcoI site

removed, and the gene32-like transcription terminator downstream of the BamHI site.

3. Ligation and Isolation of pTCpt/EGF

The 2.8kb EcoRI(blunt)-BamHI fragment of pBM16t/NDP/VGFa was ligated to the 420bp HindIII(blunt)-BamHI fragment of TacPak/EGF and the resulting DNA was used to transform competent JM109(lacIq). A correct construct was isolated by its resistance to neomycin and by DNA sequencing.

(HindIII site of pDR540)

AAGCTTACTCCC

trp-35 (16bp) lac-10
CATCCCCCTG [TTGACA] ATTAATCATCGGCTCG (TATAATG)

mRNA 5' lacI binding site lacSD
TGTGG/AATTGTG AGCGGATAACAATTCACAC {AGGA} AACAGGATCACTA

PvuI
croSD (11bp) EgIII Signal Sequence ->
{AGGA} GGTTCAGATCT ATGAAACAATCTACGATCGCCCTCGCACTTCTCC
M K Q S T I A L A L L P

EGF ->
CACTGCTGTTCACTCCAGTGACAAAAGCTAATTCTGACTCTGAATGCCCGCTGTC
L L F T P V T K A N S D S E C P L S

NsiI
TCATGACGGCTACTGCCTGCATGACGGCGTATGCATGTACATCGAAGCTCTGGAC
H D G Y C L H D G V C M Y I E A L D

SphI
AAGTACGCATGCAACTGCGTTGTTGGCTACATCGGC
K Y A C N C V V G Y I G

BamHI
GAACGTTGCCAGTACCGTGACCTGAAATGGTGGGAAGTGCCTTAAGGATCCGTGA
E R C Q Y R D L K W W E L R *

Trans. Term.

CTAATTGGGGACCCTAGAGGTCCCCTTTTTTATTTTAAAACGATC

- 5 B. Preparation of pTCPt/nVGfA ([trp-35]16bp[lac-10]
[lacSD][croSD]11bp[ATG]/ alkaline phosphatase
signal/n-terminal VGfA with sequence GTC and
GYACRC)/trans. term.-NEO)

10 This plasmid has the tac promoter elements and
uses the cro SD to express the modified VGF gene with
the N-terminal extension downstream of the alkaline
phosphatase signal sequence. The plasmid has a pBR322
background with the neomycin resistance gene.

- 15 1. Preparation of the 350bp PvuI-BamHI fragment
of pBM11/PAK/nVGfA

Plasmid pBM11/PAK/nVGfA was digested with PvuI
and BamHI and the 350bp fragment was isolated by gel
electrophoresis. This fragment contains most of the
alkaline phosphatase signal sequence and the nVGfA
20 gene.

2. Preparation of the 2.8kb PvuI-BamHI fragment
of pTCPt/EGF

Plasmid pTCPt/EGF was digested with PvuI and
BamHI and the 2.8kb fragment was isolated by gel
25 electrophoresis.

3. Ligation and Isolation of pTCPt/nVGfA

The 2.8kb fragment and the 350bp fragment were
ligated using DNA ligase and the DNA was used to
transform competent JML09(lacIq). A correct construct
30 was isolated using restriction analysis.

(HindIII site of pDR540)

AAGCTTACTCCC

trp-35 (16bp) lac-10
CATCCCCCTG [TTGACA] ATTAATCATCGGCTCG (TATAATG)

mRNA 5' lacI binding site lacSD
TGTGG/AATTGTG AGCGGATAACAATTTACAC {AGGA} AACAGGATCACTA

5

croSD (11bp) BglII Signal Sequence -> PvuI
{AGGA} GGTTTCAGATCT ATGAAACAATCTACGATCGCCCTCGCACTTCTCCC
M K Q S T I A L A L L P

10

nVGF ->
ACTGCTGTTCACTCCAGTGACAAAAGCTGACTCTGGTAACGCTATCGAAACTACT
L L F T P V T K A D S G N A I E T T

15

KpnI
CGGAAGGCGACGGCTACTGCCTGCATGCTACCTGCATCCATGCACGTGACATCGA
E G D G Y C L H G T C I H A R D I D

20

SphI EcoRI
CGGCTACGCATGCCGTTGCTCTCATGGCTACACTGGAATTCGTTGCCAGCATGTT
G Y A C R C S H G Y T G I R C Q H V

25

BamHI Trans.
GTTCTGGTTCGACTACCAGCGTTAAGGATCCGTGACTAATTGGGGACCCTAGAGGT
V L V D Y Q R *

Term.
CCCCTTTTTTATTTTAAAACGATC

30

C. Preparation of pTNPt/EGF ([trp-35]17bp[lac-10][nSD]8bp[ATG]/alkaline phosphatase signal/human EGF/trans. term.-NEO)

35

This plasmid is designed to have the tac promoter elements and utilize the N-gene SD to express human EGF behind the alkaline phosphatase signal sequence. It has a pBR322 background with the Neomycin resistance gene.

1. Preparation of 2.8kb PvuI-BamHI pTNPt

Plasmid pTNPt was digested with PvuI and BamHI and the 2.8kb fragment was isolated by gel electrophoresis.

5 2. Preparation of 300bp PvuI-BamHI fragment of pBM11/PAK/EGF

Plasmid pBM11/PAK/EGF was digested with PvuI and BamHI and the 300bp fragment was isolated.

10 3. Ligation and Isolation of pTNPt/EGF

The 2.8kb fragment and the 300bp fragment were ligated using DNA ligase and the DNA was transformed into competent JM109(LacIq). A correct construct was isolated by restriction analysis and by DNA sequencing.

15

(EcoRI site of pBM11)

GAATTACTCCCCATCC

20

trp-35 (17bp) SstI lac-10 5' lac
CCCTG [TTGACA] ATTAATCATCGAGCTCG (TATAATG) TGTGG/AATTG

25

BsmI
mRNA-> n mRNA->
TGTGAGCGGATAACAATTTACACAGCATTCAAAGCAGAAGGCTTTGGGGTGTGT
GATACGAAACGAAGCATTTGGCCGTAAGTCCGATTCCGGATTAGCTGCCAATGTGC
CAATCGCGGGGGGTTTTCGTTTCAGGACTACAACTGCCACACACCACCAAAGCTAA

30

PvuI
nSD (8bp) Signal Sequence ->
CTGAC {AGGA} GAATCCAG ATGAAACAATCTACGATCGCCCTCGCACTTCTC
M K Q S T I A L A L L

35

EGF ->
CCACTGCTGTTCACTCCAGTGACAAAAGCTAATTCTGACTCTGAATGCCCGCTGT
P L L F T P V T K A N S D S E C P L S

5 CAAGTACG CATGCAACTGCGTTGTTGGCTACATCGGCGAACGTTGCCAGTACCGT
K Y A C N C V V G Y I G E R C Q Y R

BamHI BamHI
GACCTGAAATGGTGGGAACTGCGTTAAGGATCCGTGACTAATTGGGGA
 D L K W W E L R *

Trans. Term. (BamHI site of pBM11)
CCCTAGAGGTCCCCTTTTTTATTTTAAAACGATCC

15 Example IX.
Isolation of Recombinant Polypeptides

A. Growth Factors Produced in pBM-Based Vectors Using the PL Promoter and the ts CI Repressor

20 E. coli B (HB101) containing the pBM11/NDP/
growth factor plasmids were grown in Luria Broth at
30°C. The density of the culture was measured at
550 nm and when the density reached an absorbance of
0.7 to 0.9, synthesis of the growth factor fusion
25 protein was induced by increasing the temperature to
42°C. The culture was incubated at this temperature
for 5-20 hrs, then the bacteria were isolated by cen-
trifugation and frozen at -70°C until use.

For isolation of the recombinant protein, the cells were thawed into buffer containing 0.05 M NaH_2PO_4 pH 7.2, 0.5 M NaCl, 0.01 M EDTA. One hundred fifty ml of buffer was used for a preparation from 50 g bacteria. The cells were disrupted by sonication on ice for 15 min using a 1/4-inch probe, 50% pulse at 60 watts of power. Following disruption of the cells, the insoluble protein was collected by centrifugation at 12,000

rpm in a GSA rotor for 90 min. The pellet containing the insoluble protein was then resuspended in 50 ml of 6 M guanidine hydrochloride. The insoluble material was collected by centrifugation for 2 hrs at 25,000 rpm in a Beckman-type 30 ultracentrifuge rotor. The supernatant was collected and stored at -20°C until further use.

Purification of the fusion protein was carried out on either a Sephacryl S300 or Fractogel HW-55 column equilibrated with 1 M guanidine hydrochloride. Fractions containing the fusion protein were identified as those fractions containing a polypeptide having a molecular weight consistent with the molecular weight of the polypeptide encoded by the synthetic gene as determined on a 15% polyacrylamide-urea gel.

To obtain an active form of the recombinant growth factor, the fusion protein was allowed to refold by incubating it in 50 mM Tris-HCl buffer, pH 8.7, containing 1 M guanidine hydrochloride, 1.25 mM reduced glutathione, and 0.25 mM oxidized glutathione at 4°C for 3-10 days. The biological activity of the growth factor was monitored by a competitive receptor binding assay as described above (see Example I.C). When a maximum level of activity was obtained, the protein was dialyzed against distilled water and lyophilized to dryness.

If it was desired to remove the leader sequence, the protein was cleaved either by resuspending in 70% formic acid and incubating at 40°C for 3 days or by incubating overnight at room temperature in a 100-fold molar excess of cyanogen bromide. The cleaved product was dialyzed against distilled water and lyophilized to dryness.

To further purify the recombinant growth factor, the growth factor was resuspended in 40% acetonitrile, 0.1% TFA and purified by HPLC using a BioRad TSK-250 column. Fractions containing the growth factor were pooled and further purified using reversed-phase HPLC, either Waters μ Bondapak C-18 or Rainin

Dynamax C-8. The eluant was a linear gradient of 20-40% acetonitrile containing 0.1% TFA. Fractions containing receptor binding activity were pooled, lyophilized and stored at -20°C until use.

5 1. TGF and Modified TGF

 a. N/TGF

Recombinant modified human TGF was produced from plasmid pBM11/N/TGF and contained 33 amino acids of the N-gene at the N-terminus and the sequence modification QEEK instead of the natural human sequence QEDK.

 2. Modified and Truncated VGF

 a. PAD/nVGfA

Recombinant modified VGF was produced from plasmid pBM11/PAD/nVGfA containing the extreme N-terminal sequence of VGF and the modified sequences GTC and GYACRC instead of the natural VGF sequence GDC and GMYCRC. The nVGfA fragment was expressed as a fusion protein with a modified alkaline phosphatase signal sequence at the N-terminus and was truncated at the sequence YQR at the C-terminus.

 b. NDP/VGfA

Recombinant modified VGF was produced from plasmid pBM11/NDP/VGfA beginning at the DIPAIR sequence and ending at the YKQR sequence in VGF. It has the modified sequences GTC and GYACRC instead of the natural VGF sequence GDC and GMYCRC. The VGfA fragment was expressed as a fusion protein with 32 amino acids of the N-gene at the N-terminus and the acid labile dipeptide aspartic acid-proline.

 c. VGfA

The VGF fragment was prepared as described in 2.b above and, after cleavage from the fusion protein by acid treatment, was subsequently further purified by HPLC.

d. NDP/VGFA

Recombinant modified VGF was produced from plasmid pBM11/NDP/VGFA beginning at the DIPAIR sequence and ending at the YKQR sequence in VGF and having the modified sequences GTC and GYACVC instead of the natural VGF sequence GDC and GMYCRC. The VGFA fragment was expressed as a fusion protein with 32 amino acids of the N-gene at the N-terminus and the acid labile dipeptide aspartic acid-proline.

10 3. Chimeric TGF/VGF Hybridsa. N/TTV (TGF/TGF/VGF)

Recombinant modified TTV was produced from pBM11/N/TTV and contained the amino acid sequence of human TGF in the amino terminal two-thirds of the gene with the exception of the sequence QEEK which was altered from the natural human sequence QEDK. The carboxy terminus was derived from the amino acid sequence of VGF and terminated with the sequence YQR upstream of the natural sequence PNT. The TTV fragment was expressed as a fusion protein with 33 amino acids of the N-gene at the N-terminus.

b. NDP/TTV

Recombinant TTV was produced from plasmid pBM11/N/TTV and modified as described in (a) except that the TTV fragment was expressed as a fusion protein with 32 amino acids of the N-gene at the N-terminus and the acid labile dipeptide aspartic acid-proline.

c. NDP/VTV

Recombinant modified VTV was produced from plasmid pBM11/NDP/VTV and contained the amino acid sequence of human TGF in the middle domain with the amino acid sequence QEEK replacing the natural sequence QEDK. The N-terminal and C-terminal domains were derived from the truncated VGF sequence and begin with the sequence DIPAIR and end with the sequence YQR. The VTV fragment was expressed as a fusion protein with 32

amino acids of the N-gene at the N-terminus and the acid labile dipeptide aspartic acid-proline.

d. NDP/TVV

Recombinant modified VTV was produced from
5 plasmid pBM11/NDP/TVV and contained the amino acid
sequence of human TGF in the N-terminal domain of the
gene. The middle and C-terminal domains were derived
from the truncated VGF sequence and end with the
sequence YQR. In addition, the synthetic gene has the
10 modification GYACVC for GMYCRC. The TVV fragment was
expressed as a fusion protein with 32 amino acids of
the N-gene at the N-terminus and the acid labile
dipeptide aspartic acid-proline.

15 B. Growth Factors Produced in Vectors Comprising the
tac or lac Promoters

Bacterial hosts containing expression
cassettes which comprise the tac or lac promoters were
grown at 30 to 37°C to an optical density of A600 = 0.2
20 to 0.8 in either LB broth or a chemcially defined
medium such as M9 medium supplemented with thiamine and
glucose. An appropriate antibiotic was included in the
growth medium to select for hosts containing the
expression cassette. The bacterial cultures were
25 induced with 100 to 1000 mM concentrations of IPTG and
were allowed to grow at 30°C for 16 to 24 hours. For
the expression cassettes lacking a lacI gene the
bacterial hosts carried an F-factor with the lacIq
gene, such as JM109, XL1, JM103, etc. For expression
30 cassettes which carry the lacI gene, examples of
bacterial hosts are HB101, DH1, DH5, etc. In the case
where the bacterial host has a functional lac operon
(lac+), the expression cassette can be induced with 1%
lactose. After the induction period, growth factors
35 can be isolated from either the medium or the cell
pellet.

1. PAK/EGF

Human EGF produced from the expression cassettes TacPak/EGF and pTcCpt/EGF was isolated from the medium in an active form with the alkaline phosphatase signal sequence removed. Approximately 85% of the active EGF is found in the medium, with the remainder associated with the cell pellet. These expression cassettes have yielded 4 mg/l of active EGF. The cells are removed from the medium by centrifugation and the medium is passed through an Amicon SY30, 30,000 M_r cutoff spiral filter and then passed through a Q-Sepharose column and the highly purified human EGF was eluted in 20 mM NaPO_4 pH7 with a 0 to 0.5 M NaCl gradient. Alternatively, the growth factors can be isolated from the cell pellet by osmotic shock or sonication and purified by essentially the same procedure.

2. PAK/nVGf α

Recombinant nVGf α was produced from the expression cassette pTCpt/nVGf α . The nVGf α was isolated from the cell pellet by sonication and was shown to constitute approximately 40% of the total bacterial protein.

25 C. Platelet Factor 4

Recombinant Platelet Factor 4 was isolated essentially as described above for growth factors.

1. N/PF4

Recombinant Platelet Factor 4 was produced from pBM11/N/PF4 as a fusion with the 33 N-terminal amino acids of the N-protein.

2. NDP/PF4

Recombinant Platelet Factor 4 was produced from pBM11/NDP/PF4 as a fusion with the 33 N-terminal amino acids of the N-protein and an aspartic-acid-proline cleavage site. Treatment of the fusion protein with formic acid released the mature PF4.

D. Oncostatin M

1. NDP/Oncostatin M

a. Purification of Recombinant Oncostatin M

5 Recombinant OncM fusion proteins were purified from E. coli as follows: A cell pellet from a 500 ml culture of E. coli was suspended in 40 ml of PNE buffer (0.5 M NaCl, 10 mM EDTA, 50 mM sodium phosphate), and lysed by sonication. Aggregated proteins were collected from the cell lysate by centrifugation. Aggregated proteins were sequentially extracted for 16 hrs each with 120 ml of 8 M urea solutions buffered as follows: Solution 1) 20 mM Tris, pH 5; Solution 2) 20 mM Tris, pH 8; Solution 3) 50 mM Tris, pH 11. Most aggregated proteins were solubilized by Solutions 1 and 2, while recombinant Onco M remained insoluble until treatment with Solution 3.

b. Refolding of Recombinant Molecules

20 The Solution 3 extract was then dialyzed for 24 hrs against a refolding buffer (1 M guanidine HCl, 1.2 mM oxidized glutathione, 0.2 mM reduced glutathione, 20 mM Tris HCl, pH 8.0-9.0). Lowering the pH to <pH 8.0 resulted in a 100-fold reduction in yield of biologically active OncM. Following re-folding, proteins were dialyzed versus 1 N acetic acid before testing in the growth inhibitor assay (Example I.E).

2. PAD/Oncostatin M

30 The PAD/OncoM was produced from the expression cassette pBM11/PAD/OncoM. The medium was tested for active OncoM and was shown to contain from 20 to 500 µg/l.

Example X.Biological Activity of Recombinant Growth Factors
Prepared in Procaryotic Cells5 A. EGF Receptor Binding

 This assay determines the ability of a molecule
to bind to the EGF receptor as measured by its ability to
inhibit the binding of EGF to its receptor. All growth
factors and chimeric growth factors, whether modified or
truncated, isolated to date were active in the EGF
receptor binding inhibition assay. A summary of these
results is shown below:

15

TableEGF Receptor Binding of Recombinant Growth Factors

20	<u>Peptide</u>	<u>Expression Cassette</u>	<u>Purity</u>	<u>Binds to EGF Receptor</u>
	N/TGF - modified truncated fusion	pBM11/N/TGF	95%	Yes
25	PAD/nVGfα - modified truncated fusion	pBM11/PAD/nVGfα	>95%	Yes
	NDP/VGfα - modified truncated fusion	pBM11/NDP/VGfα	>95%	Yes
30	NDP/VGfα - modified truncated fusion	pBM11/NDP/VGfα	>95%	Yes
	N/TTV - modified truncated chimeric fusion	pBM11/N/TTV	>95%	Yes
35	NDP/TTV - modified truncated chimeric fusion	pBM11/NDP/TTV	>95%	Yes

	NDP/VTV - modified truncated chimeric fusion	pBM11/NDP/VTV	>95%	Yes
5	NDP/TVV - modified truncated chimeric fusion	pBM11/NDP/TVV	>95%	Yes
	PAK/EGF	pBM11/PAK/EGF	95%	Yes
	EGF	pBM11/PAK/EGF	95%	Yes
	PAD/EGF	pBM11/PAD/EGF	95%	Yes
10	EGF	pBM11/PAD/EGF	95%	Yes
	PAK/EGF	TacPak/EGF	95%	Yes
	EGF	TacPak/EGF	95%	Yes
	PAK/EGF	pTCPt/EGF	95%	Yes
15	EGF	pTCPt/EGF	>95%	Yes

20

A comparison of the binding inhibition curves for natural mouse EGF and the bacterially expressed recombinant chimeric growth factor N/TTV (a polypeptide fusion of the 32 N-terminal amino acids of the lambda N-gene and the modified and truncated TGF/VGF hybrid) suggested that there were no differences in the binding activity.

25

B. Mitogenic Activity

30

The activity of several of the purified growth factors was tested and the activity determined in all cases was comparable to the effect caused by EGF. The compounds tested are as indicated below:

35

TableMitogenic Activity of Growth Factors

5		
	<u>Peptide</u>	<u>Mitogenic Activity*</u>
	TTV - modified truncated chimeric	Yes
10	N/TTV - modified truncated chimeric fusion	Yes
	* As measured by ^3H -thymidine or ^{125}I -IdU incorporation.	

15

C. Wound Healing1. Mid-dermal Injuries

The effect of natural or synthetic TGF, EGF and VGF, as well as recombinant growth factors on mid-dermal injuries was assessed as described in Example VE1. The percent of the original burn area which had healed was measured by computer-assisted telemetry, and the percent wound re-epithelialization was determined. Untreated wounds were approximately 15% reepithelialized. Treatment with Silvadene alone or Silvadene with EGF resulted in approximately 50% reepithelialization, while treatment with synthetic TGF or natural VGF resulted in approximately 90% re-epithelialization. The optimal concentration to promote re-epithelialization of EGF was 1-10 $\mu\text{g/ml}$, while synthetic TGF and natural VGF produced a maximal response at 0.1 $\mu\text{g/ml}$.

Experiments similar to those described above were done to test the effect in wound healing of either TGF, a modified, truncated form of VGF (VGFa), or a modified, truncated chimeric fusion of TGF and VGF (TTV), all of which were produced by recombinant technology in

Example XI.

Biological Activity of Recombinant Platelet Factor 4
Prepared in Prokaryotic Cells

The yield of recombinant Factor 4 prepared using different expression cassettes varied from about 2% to about 20% of total cell protein. These results are summarized below:

Table

Expression of PF4 in Different
Bacterial Expression Systems

	<u>Percent of Total Protein</u>
pBM11/Ngene/PF4	20%
pBM11/Ngene/DP/PF4	20%
pBM11/PAD/PF4	15%
pBM11/PF4	2%

A. Inhibition of DNA Synthesis

The highest activity seen was with the fusion protein from the plasmid pBM11/Ngene/PF4. Fifty percent of maximum inhibition of A549 cells was obtained with 0.67 µg/well.

B. Inhibition of Growth of Tumors in Nude Mice

Male nude mice were injected with platelet factor 4 or phosphate buffered saline at 2- to 3-day intervals, as described in Example 1C. As shown below, factor 4 significantly inhibited tumor growth.

Table IV

Effect of Recombinant N-Gene Platelet Factor 4
Fusion Protein on
Growth of Tumors in Nude Mice

Days Post Treatment	Tumor size (mm ³)	
	Control	Platelet Factor 4
0	17	25
6	205	25
9	275	25
14	420	40
17	545	50
20	635	85

Example XII.

Biological Activity of Recombinant Oncostatin
Prepared in Prokaryotic Cells

A. Physicochemical Characterization of Recombinant
Oncostatin M

1. SDS-PAGE

Cultures (50 ml) were grown and induced as described. Cultures were pelleted and the cell pellets were solubilized in 6 M guanidine HCl. Insoluble proteins were not removed at this point. Samples for SDS-PAGE analysis were dialyzed directly against 1 N acetic acid without refolding.

Aliquots consisting of approximately 6 µg of total bacterial protein were analyzed by SDS-PAGE on 10-20% gradient gels (5% stacking gel). Gels were stained with Coomassie Brilliant Blue, destained and dried. The apparent molecular weight M_r of the NDP-OncM fusion protein was estimated to be 32,000 by comparison of its mobility with that of standard proteins.

B. Growth Inhibitory Activity of Recombinant
Oncostatin M (N-Gene Fusion Protein)

Cellular proliferation in the presence of recombinant or native Oncostatin M was compared with proliferation in untreated samples, and expressed as a percentage of maximal (untreated) growth. Samples were assayed in duplicate or triplicate, and generally varied by less than 20% from each other. One growth inhibition assay unit of Oncostatin M is defined as the amount of protein required to cause a 50% inhibition of the growth of A375 cells seeded at 3 to 4×10^3 during a 72-hr assay. Where indicated, concentrations required for half-maximal growth inhibition were determined by extrapolation from proliferation data after transformation as follows:

$$\% \text{ Maximal Inhibition} = 100 \times \frac{1 - (A_{590}^{\text{treated}} - A_{590}^{\text{Maximal}})}{A_{590}^{\text{untreated}} - A_{590}^{\text{maximal}}}$$

20

In some cases, a modified proliferation assay was used, in which the target cell number and serum concentration were reduced. Cells were seeded at 500 cells/well in DMEM containing 5% FBS, treated with Oncostatin M in the same medium, and incubated at 37°C until untreated cells reached confluence (generally between 6 and 10 days, depending on the cell line). Monolayers were then stained and processed as described below:

30

35

Table

Growth Inhibitory Activity
of Recombinant Oncostatin M

	<u>Recombinant¹ Oncostatin M</u>		<u>Native Oncostatin M</u>	
	<u>pM</u>	<u>Percent Maximal Growth</u>	<u>pM</u>	<u>Percent Maximal Growth</u>
10	240	12.2	160	15.1
	48	19.5	32	22.2
	9.6	30.4	6.4	41.5
	1.9	70.0	1.3	67.3
	0.38	77.8	0.26	82.3
	0.08	81.5	0.05	76.0
		86.4		72.4
15		85.9		77.5
	0	100.0	0	100.0

¹Prepared using pBM11/NDP/OncoM expression cassette.

20 C. Receptor Binding Activity of Recombinant
Oncostatin M (N-Gene Fusion Protein)

25 A₅₄₉ human lung carcinoma cells were incubated with ¹²⁵I-Oncostatin M in the presence of increasing amounts of unlabeled Oncostatin M. A fraction (-3) of added ¹²⁵I-Oncostatin M bound to these cells in the absence of unlabeled Oncostatin M. When binding was measured in the presence of unlabeled native or recombinant Oncostatin M, a concentration-dependent inhibition of binding of ¹²⁵I-Oncostatin M was observed (half-maximal effect at ~300 pM). Total binding of ¹²⁵I-Oncostatin M was inhibited by approximately 90% at the highest concentration of unlabeled Oncostatin M tested. Native and recombinant Oncostatin M did not differ significantly from each other in their abilities to inhibit binding of ¹²⁵I-Oncostatin M. The results are as shown below:

TableReceptor Binding Activity
of Recombinant Oncostatin M

	<u>Recombinant¹ Oncostatin M</u>		<u>Native Oncostatin M</u>	
	<u>pM</u>	<u>Percent Maximal Growth</u>	<u>pM</u>	<u>Percent Maximal Growth</u>
10	16,000	14	27,000	12
	3,200	27	5,400	20
	640	48	1,080	40
	128	72	216	67
	26	80	43.2	81
	5.1	92	8.6	88
15	1	90	1.7	89
	0	100	0	100

¹Prepared using pBM11/NDP/OncoM expression cassette.

20

The compositions of the subject invention comprise expression cassettes for the efficient expression of polypeptides in prokaryotic cells. The expression cassettes find use in production of large amounts of polypeptides by providing for increased stability of the expression products as well as for obtaining mature folded polypeptides secreted into the growth medium of the host cell.

30

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or

35

patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it
5 will be apparent to one of ordinary skill in the art
that many changes and modifications can be made thereto
without departing from the spirit or scope of the
appended claims.

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WHAT IS CLAIMED IS:

1. An expression cassette comprising:
5 P--S.D.--met--G₁
wherein
P comprises a promoter sequence;
S.D. comprises a Shine-Dalgarno sequence;
met comprises a codon for an initiating
10 methionine;
G₁ comprises a first DNA sequence
encoding a polypeptide of interest, wherein the codons
for the N-terminal amino acids are modified using codon
degeneracy so that the nucleotides encoding said
15 polypeptide of interest approximate those of the native
nucleotide sequence associated with said S.D.
2. The expression cassette according to
Claim 1, wherein said S.D. is the N-gene Shine-Dalgarno
20 sequence.
3. The expression cassette according to
Claim 2, wherein said S.D. and said met are separated
by from about 5 to 9 nucleotides.
25
4. An expression cassette comprising:
P--S.D.--met--L--G
wherein
P comprises a promoter sequence;
30 S.D. comprises a Shine-Dalgarno sequence;
met comprises a codon for an initiating
methionine;
L comprises a first DNA sequence encoding
a leader sequence wherein the codons for the amino
35 acids are modified using codon degeneracy so that the
nucleotides encoding said leader sequence approximate

those of the native nucleotide sequence associated with said S.D.; and

G comprises a second DNA sequence encoding a polypeptide of interest.

5

5. The expression cassette according to Claim 4, wherein the first about 7 to 30 codons of said first DNA sequence comprise modified codons.

10

6. The expression cassette according to Claim 5, wherein said S.D. comprises the N-gene Shine-Dalgarno sequence and said leader sequence comprises an alkaline phosphatase signal sequence.

15

7. The expression cassette according to Claim 4, wherein said leader sequence is a an N-terminal amino acid sequence from a highly expressed gene; a hydrophobic amino acid sequence; or a hydrophilic amino acid sequence.

20

8. The expression cassette according to Claim 7, wherein said highly expressed gene is the bacteriophage lambda N-protein gene or Cro gene, or the bacterial beta-galactosidase gene.

25

9. The expression cassette according to Claim 7, wherein said hydrophobic amino acid sequence is the bacterial alkaline phosphatase signal sequence.

30

10. The expression cassette according to Claim 7, wherein said hydrophilic amino acid sequence is about 41 N-terminal amino acids from amphiregulin.

35

11. An expression cassette comprising:

P--S.D.--met--L--G

wherein

P comprises a bacteriophage lambda P_L promoter; a bacterial lac promoter; or a bacterial trp-lac fusion promoter.

5 S.D. comprises a Shine-Dalgarno sequence; met comprises a codon for an initiating methionine;

L comprises a first DNA sequence encoding a leader sequence; and

10 G comprises a second DNA sequence encoding a polypeptide of interest.

12. An expression cassette according to Claim 11, wherein said promoter further comprises a regulatory sequence.

15 13. An expression cassette according to Claim 12, wherein said regulatory sequence comprises a bacteriophage lambda O_L operator; or a bacterial lac operator.

20 14. An expression cassette according to Claim 11, wherein said S.D. comprises an N-gene or a Cro gene ribosomal binding site.

25 15. An expression cassette according to Claim 11, wherein said leader sequence comprises about 8 to about 35 N-terminal amino acids from a bacteriophage lambda N-gene or Cro gene; or a bacterial alkaline phosphatase gene.

30 16. An expression cassette according to Claim 11, further comprising at least one codon joined in reading frame between said first DNA sequence and said second DNA sequence.

35

17. An expression cassette according to Claim 16, wherein said codons encode a chemical cleavage site or an enzymatic cleavage site.

5 18. An expression cassette according to Claim 17, wherein said chemical cleavage site is aspartic acid-proline.

19. An expression cassette comprising:

10 P--S.D.--met--L--G

wherein

P comprises a promoter having a -35 and a -10 regulatory sequence;

S.D. comprises a Shine-Dalgarno sequence;

15 met comprises a codon for an initiating methionine;

L comprises a first DNA sequence encoding an alkaline phosphatase signal sequence, wherein the codons for the amino acids of said signal sequence
20 approximate those of the native nucleotide sequence associated with said S.D.; and

G comprises a second DNA sequence encoding a polypeptide of interest.

25 20. The expression cassette according to Claim 19, wherein said -35 and said -10 regulatory sequences are substantially the same as those from the bacterial lac promoter.

30 21. The expression cassette according to Claim 20, wherein said regulatory sequences are from the bacterial lac promoter.

35 22. The expression cassette according to Claim 19, wherein said -35 regulatory sequence is substantially the same as the -35 regulatory sequence from the trp promoter and the -10 regulatory sequence

is substantially the same as the -10 regulatory sequence from the lac promoter.

23. The expression cassette according to
5 Claim 22, wherein said -35 regulatory sequence is from the trp promoter and said -10 regulatory sequence is from the lac promoter.

24. The expression cassette according to any
10 one of Claims 21 and 23 wherein said S.D. comprises the N-gene Shine-Dalgarno sequence.

25. The expression cassette according to
Claim 23, wherein said S.D. comprises the Cro gene
15 Shine-Dalgarno sequence.

26. The expression cassette according to
Claim 25, wherein said S.D. is separated from said met
by about 11 or 12 nucleotides.
20

27. The expression cassette according to
Claim 24, wherein said S.D. is separated from said met
by about 7 or 8 nucleotides.

25 28. An expression cassette comprising:

P--S.D.--met--L--G--T

wherein

P comprises a promoter having a -35
regulatory sequence from the trp promoter and a -10
30 consensus regulatory sequence from the lac promoter;
S.D. comprises a Cro gene Shine-Dalgarno
sequence;

met comprises a codon for an initiating
methionine;

35 L comprises a first DNA sequence encoding
an alkaline phosphatase signal sequence, wherein the
codons for the amino acids are modified using codon

degeneracy so that the nucleotides encoding said signal sequence approximate those of the native nucleotide sequence associated with an N-gene Shine-Dalgarno sequence;

5 G comprises a second DNA sequence encoding a polypeptide of interest; and

T comprises a transcriptional termination region substantially similar to the gene 32 transcriptional termination region.

10

29. An expression cassette comprising:

P--S.D.--met--L--G--T

wherein

15 P comprises a promoter having a -35 regulatory sequence from the trp promoter and a -10 regulatory sequence from the lac promoter;

S.D. comprises an N-gene Shine-Dalgarno sequence;

20 met comprises a codon for an initiating methionine;

L comprises a first DNA sequence encoding an alkaline phosphatase signal sequence, wherein the codons for the amino acids are modified using codon degeneracy so that the nucleotides encoding said signal sequence approximate those of the native nucleotide sequence associated with said S.D.;

25 G comprises a second DNA sequence encoding a polypeptide of interest; and

30 T comprises a transcriptional termination region substantially similar to the transcriptional termination region of gene 32.

30. An expression cassette comprising:

P--S.D.--met--L--G--T

35 wherein

P comprises a promoter having a -35 regulatory sequence and a -10 consensus regulatory

sequence from the lac promoter;

S.D. comprises a N-gene Shine-Dalgarno sequence;

5 met comprises a codon for an initiating methionine;

L comprises a first DNA sequence encoding an alkaline phosphatase signal sequence, wherein the codons for the amino acids are modified using codon degeneracy so that the nucleotides encoding said signal
10 sequence approximate those of the native nucleotide sequence associated with said S.D.;

G comprises a second DNA sequence encoding a polypeptide of interest; and

T comprises a transcriptional termination
15 region substantially similar to the transcriptional termination region of gene 32.

31. An expression cassette comprising:

P--S.D.--met--L--G--T

20 wherein

P comprises a promoter having a -35 consensus regulatory sequence from the trp promoter and a -10 consensus regulatory sequence from the lac promoter;

25 S.D. comprises a Cro gene Shine-Dalgarno sequence;

met comprises a codon for an initiating methionine;

L comprises a first DNA sequence encoding
30 an alkaline phosphatase signal sequence, wherein the codons for the amino acids are modified using codon degeneracy so that the nucleotides encoding said signal sequence approximate those of the native nucleotide sequence associated with said the N-gene Shine-Dalgarno
35 sequence;

G comprises a second DNA sequence encoding a polypeptide of interest; and

T comprises a transcriptional termination region from gene 32.

5 32. A prokaryotic cell comprising an expression cassette according to Claims 28 to 31.

33. A cell according to Claim 32, wherein said cell is an E. coli cell.

10 34. A fusion protein comprising a polypeptide of interest and about 8 to about 35 N-terminal amino acids from a bacteriophage lambda N-protein or Cro protein or a modified bacterial alkaline phosphatase signal sequence joined to the N-terminus of said
15 polypeptide of interest.

35. A fusion protein according to Claim 34 further comprising a central region of at least one amino acid between said polypeptide of interest and
20 said N-terminal amino acids.

36. A fusion protein according to Claim 35, wherein said central region comprises a chemical cleavage site or an enzymatic cleavage site.
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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03872

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC: C12P 21/00, C12P 19/34, C12N 15/00, C12N 1/20; C07H 15/12; C07K 13/00 U.S. CL.: 435/68, 91, 172.1, 172.3, 253, 320; 536/27; 530/350		
II. FIELDS SEARCHED Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	435/68, 91, 170, 172.1, 172.3, 253, 320 536/27 530/250+ 935/6	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁸		
Chemical Abstract Data Base (CAS) 1967-1989; BIOSIS, DATABASE 1969-1989 Keywords: plasmid, vector, colE1 origin, cI857 repressor, PL lambda promoter, ribosomal binding site, N gene, see attachment		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	U.S., A, 4,543,329 (DAUM ET AL) 24 September 1985. See abstract column 1.	17, 18 and 36
Y	Chemical Abstracts, Volume 103, No. 7, issued 1985, August 19 (Columbus, Ohio, U.S.A), F. Marcus, "Preferential cleavage of aspartyl- prolyl peptide bonds in dilute acid", See page 616, column 2, the abstract no. 5444m. Int. J. Pept. Protein Res. 1985. 25(5), 542-546 (Eng).	18
Y	THE EMBO JOURNAL, Volume 1, issued 1982 December (Oxford, U.K.) (M. ZABEAU ET AL), "Enhanced expression of cro-beta-galactosidase fusion proteins under the control of the Pr promoter of bacteriophage lambda", See pages 1217-1224, See particularly pages 1217 and 1221.	1-26
[*] Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 26 January 1989		Date of Mailing of this International Search Report 07 MAR 1989
International Searching Authority ISA/US		Signature of Authorized Officer Richard C. Peet RICHARD C. PEET

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	U.S., A, 4,582,800 (ROBERT M. CROWL), 15 April 1986, See abstract and columns 3, 4 and 9.	1-3 4-36
P,Y	U.S., A, 4,711,845 (GELFAND ET AL), 08 December 1987, See abstract and columns 2, 3 and 7.	1-36
Y	U.S., A, 4,565,785 (GILBERT ET AL), 21 January 1986, See abstract and columns 1, 2, 6 and 7.	4-36
Y	CHEMICAL ABSTRACTS, Volume 103, no. 3, issued 1985, July 22 (Columbus, Ohio, U.S.A), T.Miyake et al, "Secretion of human interferon-alpha induced by secretion vectors containing a promoter and signal sequence of alkaline phosphatase gene of Escherichia coli". See page 153, column 2, the abstract no. 17753r J. Biochem. (Tokyo), 1985, 97(5), 1429-36 (Eng).	4-36
X Y	CHEMICAL ABSTRACTS, Volume 104, no. 7, issued 1986, February 17 (Columbus, Ohio, U.S.A), D.H. Gelfand et al, "Monitoring and control systems for recombinant manipulations", See page 149, column 2, the abstract no. 46697b, PCT Int. Appl. WO 85 03,522	1-3 4-36
Y	CHEMICAL ABSTRACTS, Volume 106, no. 17, issued 1987, April 27, (Columbus, Ohio, U.S.A.), S. Chang et al., "Alkaline phosphatase mediated processing and secretion of recombinant proteins, DNA sequences for use therein and cells transformed using such sequences". See page 149, the abstract no. 133062a, Eur. Pat. Appl. EP 196,864	4-36